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ORIGINAL PAPER

A comparative study of bioactive secondary metabolite production in diploid and tetraploid *Echinacea purpurea* (L.) Moench

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Abstract The impact of the ploidy level on biomass accumulation and the production of high-value secondary metabolites was studied in *Echinacea purpurea* (L.) Moench. Tetraploid *E. purpurea* was obtained by treating diploid explants with colchicine. The morphology, biomass yield, the contents of caffeic acid derivatives and alkamides, and the activities of phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) were compared between diploid plants and tetraploid plants of *E. purpurea*. The total fresh root weight and total dry root weight of the tetraploid plants were 39.32 and 40.48 % higher than those of the diploid plants, respectively. The chemical profiles of the diploid and tetraploid *E. purpurea* plants were similar, as determined through a comparison of their FTIR spectra and second derivative spectra. The caffeic acid derivatives and alkamides in the diploid and tetraploid plants were determined by HPLC. The tetraploid plants had higher contents of both of these types of molecules. In addition, the tetraploid plants had higher PAL and C4H activities compared with the diploid plants. The enhancement in the PAL and C4H activities was accompanied with an increase in the cichoric acid content, which indicates that the induction of polyploidy in *E. purpurea* resulted in higher PAL and C4H expression and promoted the biosynthesis of cichoric acid. Therefore, the induction of polyploidy may be a valid strategy to achieve a higher yield of biomass and bioactive compounds in *E. purpurea*.

Keywords Cichoric acid · Alkamides · Ploidy level · *Echinacea* · Phenylalanine ammonia-lyase · Cinnamate 4-hydroxylase

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Introduction

Polyploidy is frequently accompanied by conspicuous changes in morphology and metabolite production (Dhawan and Lavania 1996; Kaensaksiri et al. 2011; Lavania 2005; Nilanthi et al. 2009; Sun et al. 2011; Van Laere et al. 2011). In particular, polyploidy may increase the content of bioactive secondary metabolites in medicinal plants (Berkov and Philipov 2002; Dehghan et al. 2012; Dhawan and Lavania 1996; Gao et al. 1996; Lavania et al.2012). Similarly, the impact of polyploidy on biomass is generally positive, but sometimes this may be negative depending upon the metabolic cost of metabolite being produced i.e. longer the metabolic pathaway larger is the negative effect of polyploidy on biomass (Lavania et al. 2012). Ploidy level manipulation has been suggested as an interesting approach for increasing both plant biomass accumulation and the production of high-value plant secondary metabolites (Lavania 2005). Thus, the induction of polyploidy is a strategy to achieve a higher yield of bioactive chemicals.

Echinacea purpurea (L.) Moench, which is an herb native to North America, is well known for its effects on immune

modulation and is widely used as a dietary supplement, functional food ingredient, and food additive (Lindenmuth and Lindenmuth 2000; Barrett 2003; Hall 2003). Caffeic acid derivatives and alkamides are considered the major bioactive compounds of *E. purpurea* (Barnes et al. 2005). Phenolic compounds are derived from phenylalanine through the core phenylpropanoid pathway by the action of phenylalanine ammonia-lyase (PAL) and cinnamate-4-hydroxylase (C4H), which are responsible for the hydroxylation of cinnamic acid to *p*-coumaric acid (Pina et al. 2012). The PAL and C4H activities are positively correlated to phenylpropanoid product accumulation (Bate et al. 1994; Bi et al. 2007; Schilmiller et al. 2009). Tetraploid *E. purpurea* has been obtained by treating diploid petiole explants with colchicine (Nilanthi et al. 2009). However, the impact of the ploidy level on the biosynthesis and accumulation of caffeic acid derivatives and alkamides has not yet been studied.

In this work, ploidy manipulation was used to obtain tetraploid *E. purpurea*. The caffeic acid derivatives and alkamides contents of diploid ($2n = 22$) and induced tetraploid ($4n = 44$) *E. purpurea* were compared. In addition, the impact of the ploidy level on caffeic acid derivatives biosynthesis was studied through the determination of the activities of phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H).

Materials and methods

Plant source

Primary *E. purpurea* seeds were supplied by the Company of Plantation Products (Norton, MA, USA) and were cultivated in the Garden of Chinese Medicinal Plants on the campus of South China Agricultural University. The offspring seeds were used in this study.

Induction of tetraploids and determination of ploidy level

Tetraploid *E. purpurea* was obtained by treating diploid petiole explants with colchicine. The petiole explants were precultured on shoot regeneration medium for 1 week to heal the cutting wound and initiate cell division and then transferred to shoot regeneration medium with different concentrations (0, 30, 60, 120, or 240 mg/L) of colchicine for 30 days or with 120 mg/L colchicine for different time periods (7, 14, 21, or 28 days). Eight explants were cultured in one bottle. After colchicine treatment, the treated explants were transferred to shoot regeneration medium and cultured for 40 days. The regenerated shoots were cut from the mother tissues and cultured on rooting medium for the initiation of roots and further growth of the intact regenerated plantlets. The determination of the ploidy level was based on the observation of the chromosome number. The detailed procedures are described in our previous study (Nilanthi et al. 2009).

Cultivation of diploid and tetraploid

Diploid seedlings germinated from seeds and tetraploid plantlets obtained from the tissue culture with similar size and developmental stage, as determined by their morphological features, were cultivated in the Teaching and Research Base of South China Agricultural University in April 2011. The 2-year-old plants were harvested for observation and analysis in April 2013.

Observation of the morphological features of the root system

Three healthy diploid plants and three healthy tetraploid plants were randomly selected, and their aerial parts were removed. Their root systems with earth were soaked in water for 12 h. The earth was slowly rinsed off by flowing water. The roots that fell off were carefully collected by a sieve. The root system was scanned using a WinRhizo Pro LA2400 instrument (Regent Instruments, Quebec, Canada). The morphological features of the root axis as an organ, including the root length, root surface area, root volume, and root mean diameter, were evaluated using a computer image analysis software (WinRHIZO-2004a, Regent Instruments, Quebec, Canada). After morphological analysis, the fresh weight of the root system was measured. It was then heated at 105 °C in an oven until a constant weight was obtained, and the dry weight was recorded.

Chemical profile analysis using FTIR

The flowers, stems, leaves, roots, and rhizomes of the diploid and tetraploid plants were dried with hot air at 40 °C for 48 h and pulverized. The powder was sieved using a 200 mesh sieve. Accurately weighed 1 mg of dry sample powder was well mixed into 200 mg of dry KBr powder in an agate mortar. The mixture was ground to a fine powder, transferred into a pellet-forming die, and pressed to form a transparent pellet. The pellet was measured at 25 °C in an atmosphere with 30 % relative humidity using a VERTEX70 FTIR spectrometer (Bruker Optics, Ettlingen, Germany) over a region from 4,000 to 400 cm^{-1} with a resolution of 4 cm^{-1}. The background was determined from a pellet of KBr that contained no sample and subtracted from the sample spectra. Five replicate pellets were prepared for each, and each sample was measured four times. The mean of the 20 spectra obtained for each sample was used for analysis and comparison.

Table 1 Morphological data from different parts of diploid and tetraploid plants

Plant ploidy	Plant height (cm)	Rhizome diameter (cm)	Leaf index (length/width; cm)	Mean root diameter (mm)	Stem diameter (mm)
Diploid	48.6 ± 4.7a	5.38 ± 0.75b	2.81 ± 0.2a	0.76 ± 0.15b	0.72 ± 0.16b
Tetraploid	35.2 ± 6.7b	6.94 ± 0.87a	2.42 ± 0.1b	1.07 ± 0.22a	1.21 ± 0.21a

The values in each vertical column that are followed by different letters are significantly different

Determination of caffeic acid derivatives and alkamides

Accurately weighed 0.1 g of the sample powder (described in Chemical profile analysis using FTIR) was extracted with 20 ml of 70 % ethanol for 20 min assisted by ultrasonic treatment (40 kHz). The extract solution was centrifuged for 10 min at a speed of 3,500 rpm (Eppendorf 5804R, Germany), and the supernatant was collected. The precipitate was extracted again, and the supernatants were combined. The solvent of the supernatant was evaporated under vacuum. The residue was reconstituted with 10 ml of 70 % ethanol. The solution was filtered through a 0.45-μm microporous membrane, and the filtrate was used as the sample solution for the determination of caffeic acid derivatives. The sample solution for the determination of alkamides was prepared using the same procedure as above with the exception that the 70 % ethanol was replaced by methanol.

Cichoric acid, caffeic acid, and chlorogenic acid were dissolved in appropriate volume of 70 % ethanol and diluted to obtain a solution of 0.0400 mg/ml.

The HPLC determination was conducted on a LC 20A system equipped with a LC-20A-series solvent delivery pump, SPD-M20A PDA detector, SIL-20A autosampler, and an LCSolution workstation (Shimadzu, Japan). A Gemini C18 reversed-phase column (4.6 mm × 250 mm, 5 μm, Phenomenex, USA) was used. Water containing 0.1 % formic acid (A) and acetonitrile (B) was used as the mobile phase. A gradient elution at a flow rate of 1.0 ml/min was programmed as follows: 0–9 min, 10–18.5 % B; 9–9.5 min, 18.5–45 % B; 9.50–39.50 min, 45–80 % B; 39.5–42.0 min, 80–100 % B; 42.0–45.0 min, 100–10 % B. The UV spectra were recorded in the range from 200 to 400 nm, and 330 and 254 nm were used for the quantification of caffeic acid derivatives and alkamides, respectively (Luo et al. 2003). The HPLC determination of each sample was conducted in triplicate.

The main peaks of alkamides on the HPLC chromatograms were identified by comparing the UV spectra and ESI/MS/MS data with literature data. The ESI/MS/MS data of a peak was acquired through the off-line coupling of HPLC to ESI–MS as follows: The eluate of the peak was collected from the HPLC outlet during the corresponding elution time and was introduced into a Finnigan TSQ Quantum triple-quadrupole mass spectrometer (Thermo Electron Corporation, USA) equipped with an ESI source through a syringe pump at a flow rate of 10 μl/min. The alkamides were determined in the positive ion mode. The automatic compound optimization procedure of the Xcalibur 2.0 workstation (Thermo Electron Corporation, USA) was used to fine-tune and optimize the mass spectrometer for the compound in the ESI/MS/MS mode. The ESI/MS/MS data were then acquired using the Tune Master Procedure.

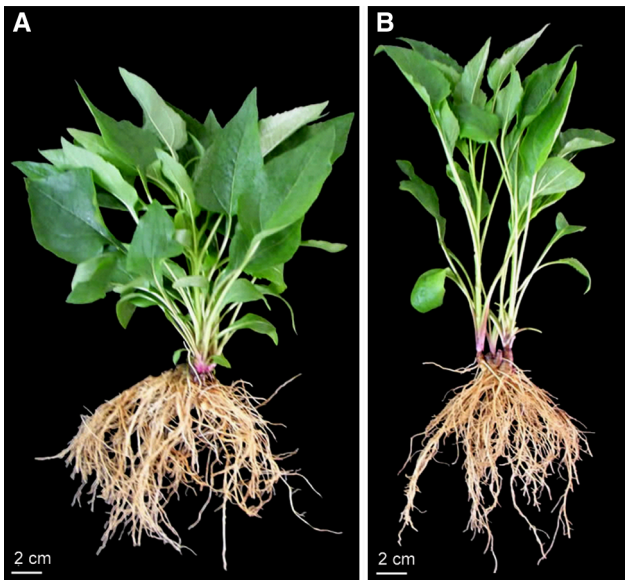

Fig. 1 Image of tetraploid (**a**) and diploid (**b**) plants

Determination of PAL and C4H activities

Accurately weighed 1 g of fresh *Echinacea* leaves or roots and rhizomes was homogenized in 2 ml of potassium phosphate buffer (200 mM, pH 7.5, containing 2 mM 2-mercaptoethanol). After filtration and centrifugation (15 min at 10,000×*g*), the supernatant was diluted 20-fold and used for enzymatic analysis.

The PAL activity was determined spectrophotometrically using the method described by Edwars and Kessmann (1992), which is based on the rate of the conversion of L-phenylalanine into trans-cinnamic acid measured by the absorbance at 290 nm. The absorbance of 0.5 ml of enzyme extract, 2 ml of 50 mM Tris–H_2SO_4 buffer (pH 8.8), and 1 ml of 20 mM L-phenylalanine was measured before and after the incubation of the mixture in water at

Fig. 2 Image of root system of tetraploid (**a**) and diploid (**b**) plants

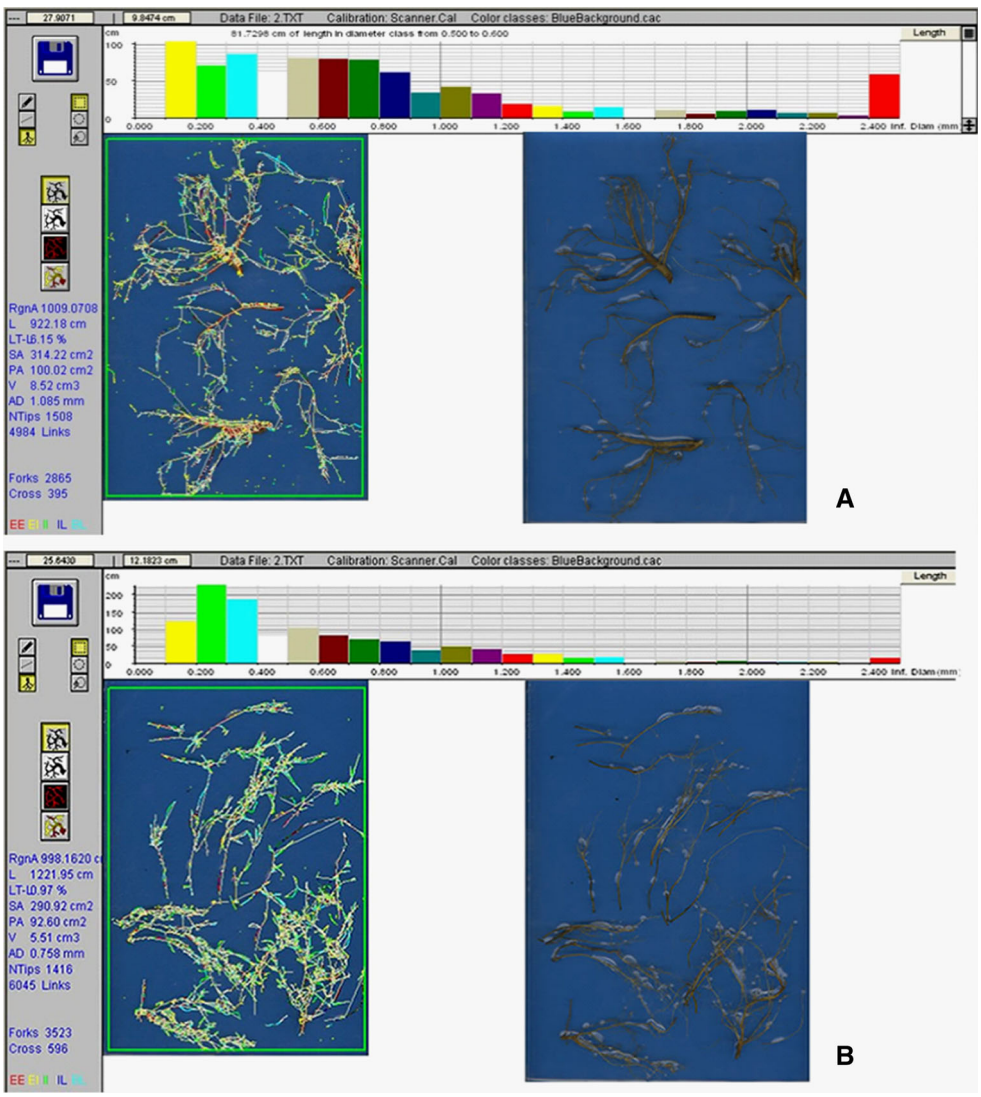

Fig. 3 Image analysis of the root system of tetraploid (**a**) and diploid (**b**) plants

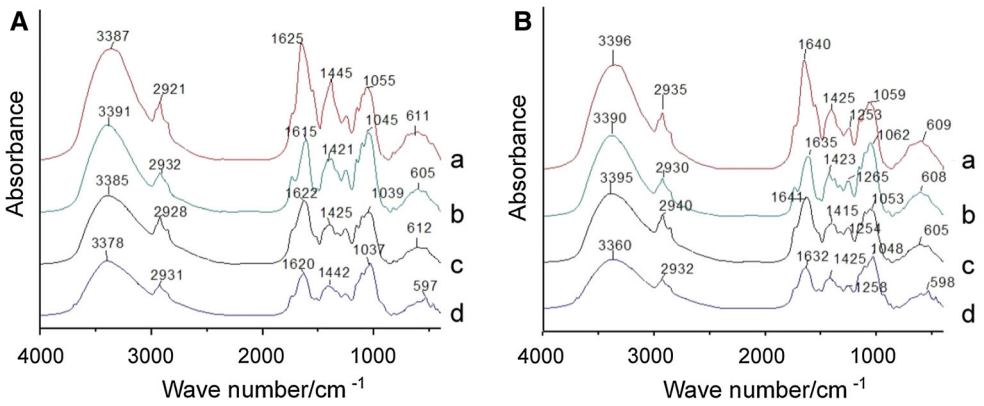

Fig. 4 FTIR spectra of the flowers (*a*), stems (*b*), leaves (*c*), and roots and rhizomes (*d*) of diploid (**a**) and tetraploid (**b**) plants

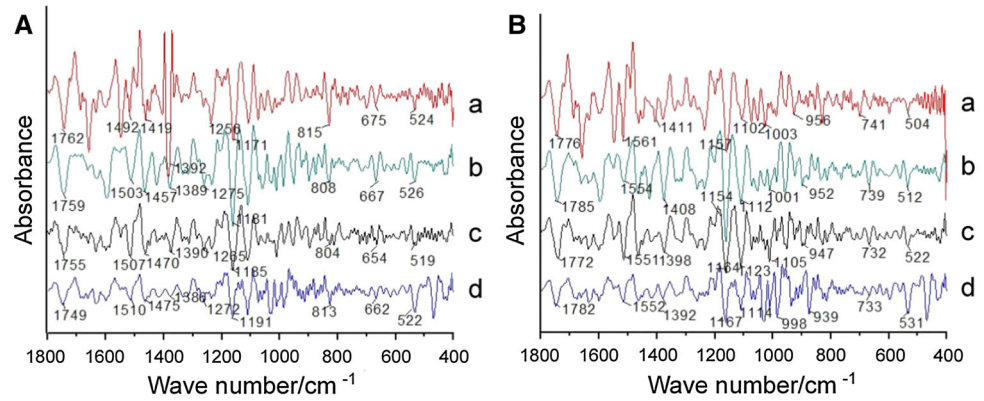

Fig. 5 Second derivative FTIR spectra of the flowers (*a*), stems (*b*), leaves (*c*), and roots and rhizomes (*d*) of diploid (**a**) and tetraploid (**b**) plants

40 °C for 15 min. The enzyme activity was stopped by the addition of 0.1 ml of 6 M HCl. One unit of activity was defined as the amount of extract that catalyzes the production of cinnamic acid and increases the absorbance by 0.01 per hour, and PAL activity was expressed as units per gram per hour.

The C4H activity was assayed using the method described by Lamb and Rubery (1975) with a slight modification. The extract (0.2 ml) was added to 2 ml of reaction buffer (50 mM phosphate buffer containing 2 mM 2-mercaptoethanol, 2 mM trans-cinnamic acid, and 0.5 mM NADPH). The solution was incubated for 1 h at 37 °C. The reaction was stopped through the addition of 0.1 ml of 6 M HCl and readjusted to pH 11 with 6 M NaOH. The absorbance at 340 nm was measured before and after the incubation of the mixture. One unit of activity was defined as the amount of extract that catalyzes the production of p-hydroxyl-cinnamic acid and increases the absorbance by 0.01 per h. The C4H activity was expressed as units per gram per hour.

Statistical analysis

The statistical analysis of the morphological and chemical results was performed using the SPSS 21 software (IBM, USA). The measured values are expressed as the mean ± standard deviation (SD) from three independent experiments ($n = 3$). The unpaired Student's t test at a probability of 5 % was used to compare the obtained data to determine the differences between the diploid and tetraploid plants.

Results

Comparison of morphological features of diploid and tetraploid plants

Tetraploid plants were more "stocky" than diploid plants. The tetraploid plants were shorter than the diploid plants, the stem diameter of the tetraploid plants was larger than that of the diploid plants, and the tetraploid plants were found to have shorter but broader leaves compared with the diploid plants (Table 1; Fig. 1). Compared with the diploid plants, the tetraploid plants had a higher number of thick roots, a shorter total root length, and fewer lateral roots (Fig. 2). Through a root system analysis (Fig. 3), the total root length of tetraploid plants was found to be 77.27 % of that of diploid plants; however, the total root surface area, total root volume, root mean diameter, total fresh root weight, and total dry root weight were 9.47, 55.17, 40.79,

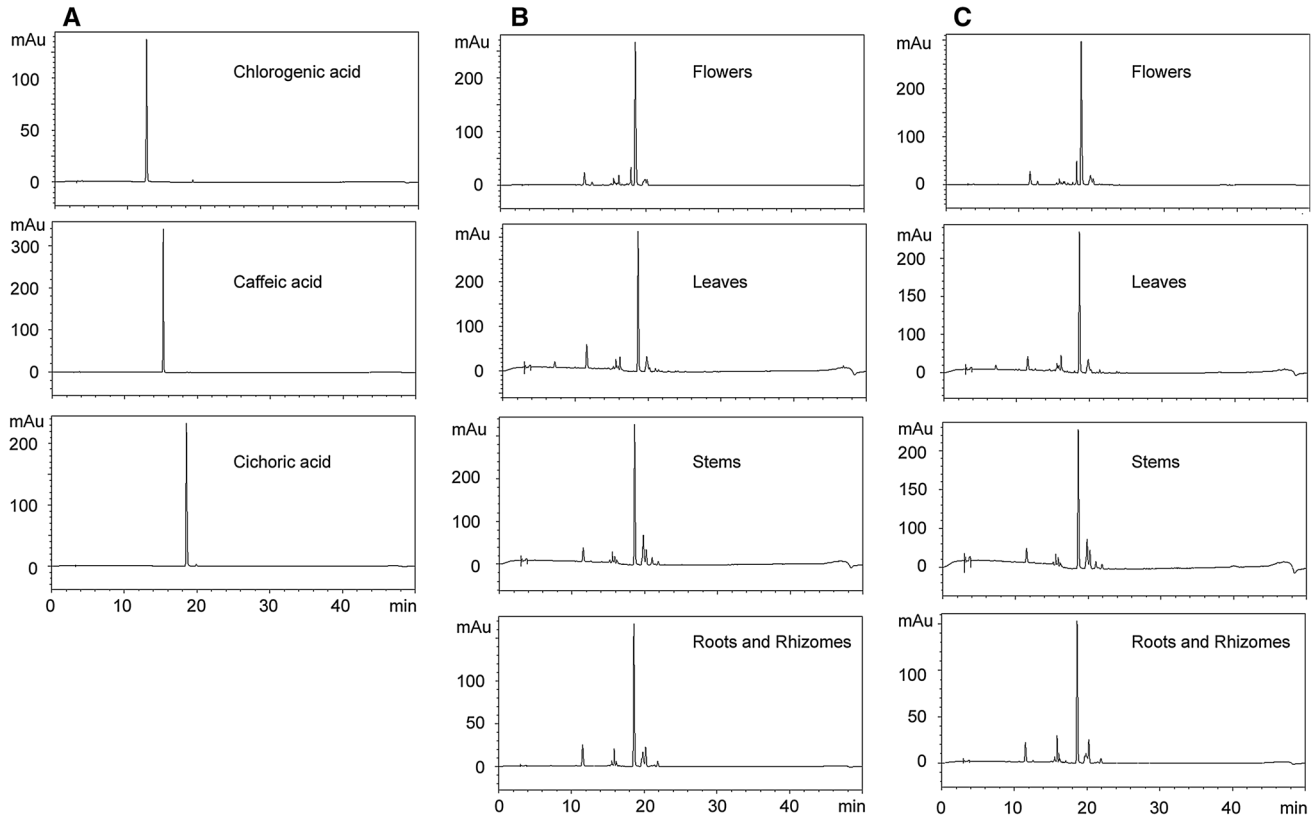

Fig. 6 HPLC chromatograms detected at 330 nm: reference standards (**a**), diploid plant (**b**), and tetraploid plant (**c**)

Table 2 Cichoric acid contents (mg/g) in different parts of diploid and tetraploid plants

Plant ploidy	Flowers	Leaves	Stems	Roots and rhizomes
Diploid	8.29 ± 1.12a	7.38 ± 0.85a	0.67 ± 0.06b	10.51 ± 1.21b
Tetraploid	10.38 ± 1.21a	8.94 ± 0.97a	0.82 ± 0.05a	13.43 ± 1.17a

The values in each vertical column that are followed by different letters are significantly different

Table 3 The identification of three alkamide compounds

Symbol	Rt	$[M + H]^+$	UV λ_{max}	Compound name
1	26.84	230.70	255	udeca-2Z,4E-diene-8,10-diynioc acid isobutylamide
2	31.62	244.77	260	dodeca-2Z,4E-diene-8,10-diynioc acid isobutylamide or udeca-2Z,4E-diene-8,10-diynioc acid 2-methylbutylamide
3	41.80	248.97	260	dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide or dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide

39.32, and 40.48 % higher than those of the diploid plants, respectively. According to the distribution diagram of the root length and root diameter (Fig. 3), the length of the roots of tetraploid plants with a diameter greater than 1.4 mm was markedly longer than that of diploid plants, and the length of the roots of tetraploid plants with a diameter greater 2.4 mm was significantly longer than that of the diploid plants. This result indicates that the root system of tetraploid plants was more developed.

Chemical profile analysis using FTIR

The FTIR spectra and second-derivative spectra of the flowers, stems, leaves, and roots and rhizomes of 2-year-old diploid plants and tetraploid plants are shown in Figs. 4 and 5. The peak at 3,390 cm^{-1} displays the characteristic absorptions of the O–H stretching vibrations of polysaccharides, glycosides, and polyphenols. The C–H stretching vibrations at 2,930 cm^{-1} and the C–H bending vibration at 1,420 cm^{-1} indicate the presence of unsaturated alkyls in the sample. The peak at 1,630 cm^{-1} displays the

characteristic absorptions of the O–H bending vibration of water, the COO^- asymmetrical stretching vibration of organic acids and amino acid residues, and the stretching vibration of the benzene ring of aromatic compounds, which was confirmed by the high contents of polyphenols and alkamides in *E. purpurea*. The peak at 1,250 cm^{-1} displays the characteristic absorptions of the C=C, C=O–C, and P=O stretching vibrations of polysaccharides, glycosides, and phospholipids. The peak at 1,050 cm^{-1} displays the characteristic absorptions of the C–OH bending vibrations of polysaccharides and glycosides.

The spectra of different parts of the diploid plants and tetraploid plants shared similar peaks, which indicates that their chemical profiles were similar. Of these, the spectra of the flowers were more similar to those of the leaves, and the spectra of the stems were more similar to those of the roots and rhizomes.

Because herbal medicine is a mixture of chemicals and the FTIR spectrum consists of the overlapped spectra of many chemicals, some features will be unnoticeable on the original FTIR spectrum. Therefore, a second derivative spectrum of the fingerprint region, namely from 1,800 to 400 cm^{-1}, was used to further compare the chemical profile of the different samples. As shown in Fig. 5, the spectra of different samples exhibit a similar profile. However, these spectra showed more differences than the original spectra. The region from 1,600 to 1,450 cm^{-1} displays the stretching vibration of the benzene ring of aromatic compounds. In this region, the spectra of different parts of the plants showed an obvious difference. The spectra of the flowers and leaves showed a stronger absorption, which indicates that these parts have higher contents of aromatic compounds.

Determination of caffeic acid derivatives and alkamides

Caffeic acid derivatives are the main bioactive compounds in *E. purpurea* and are conventionally used as marker compounds for quality control. Therefore, their contents in diploid plants and tetraploid plants were compared. Fig. 6 shows the HPLC chromatograms of caffeic acid derivatives of different samples. Different parts of the diploid and tetraploid plants shared similar peaks, which indicates that these samples contained similar caffeic acid derivatives. Little chlorogenic acid and no caffeic acid were detected in all of the samples. Cichoric acid accounted for most of the contents of caffeic acid derivatives. Therefore, the cichoric acid content was used as a marker of caffeic acid derivatives. Its contents in the different samples are listed in Table 2. The results show that the cichoric acid contents of the flowers, leaves, stems, and roots and rhizomes of tetraploid plants were 125.2, 121.1, 122.4, and 127.8 % of those of the diploid plants, respectively. It has been reported that the phenylpropanoid content is generally significantly higher in tetraploids compared with diploid *S. commersonii* (Caruso et al. 2011). Therefore, it appears that the induction of polyploidy generally increases the phenylpropanoid content in plants. The underlying mechanisms are worthy of further study.

Alkamides are the other class of bioactive compounds in *E. purpurea*. There are many alkamide compounds in *E. purpurea* plants, and their contents are very low. Their high-purity reference standards for quantitative determination are not easily available. Therefore, the peaks of alkamides on the HPLC chromatograms were putatively identified by comparing the UV spectra and ESI/MS/MS data with literature data (Luo et al. 2003), and the alkamide contents in different parts of the diploid and tetraploid plants were compared based on the total peak area of the identified peaks per gram. Three main alkamides at the retention times (Rts) of 26.84, 31.62, and 41.80 min were putatively identified (Table 3; Fig. 7).

Figure 8 shows the HPLC chromatograms of the alkamides present in the different samples. The different parts of the diploid plants and tetraploid plants shared similar

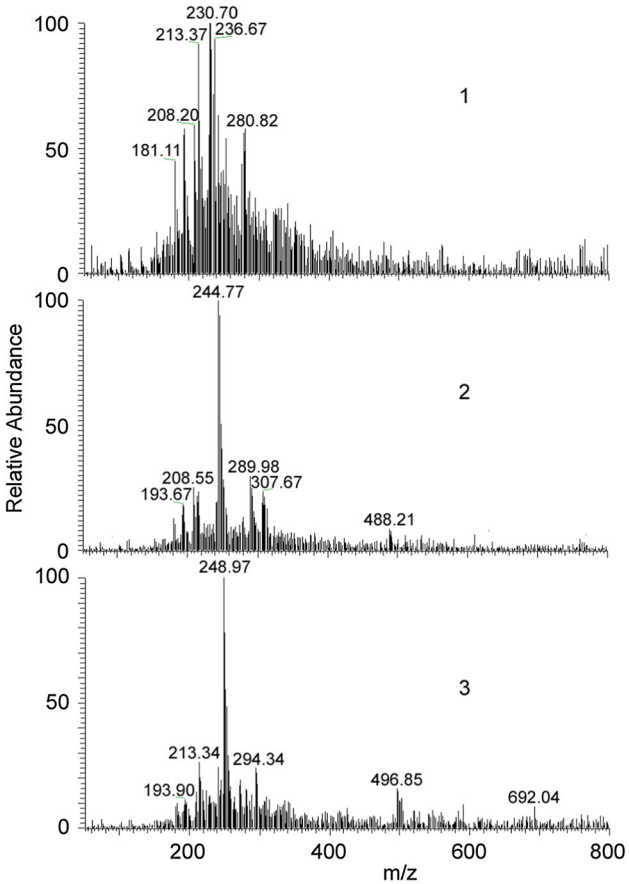

Fig. 7 Mass spectra of the identified alkamide compounds

peaks, which indicates that these parts contain similar alkamides. The alkamide contents of the flowers, leaves, stems, and roots and rhizomes of tetraploid plants were 119.57, 117.64, 121.24, and 123.57 % of those of diploid plants, respectively (Table 4).

PAL and C4H activities of diploid and tetraploid plants

Caffeic acid derivatives are more commonly known as phenylpropanoids, which are synthesized from the amino acid phenylalanine (Dixon and Pavia 1995). Phenylalanine

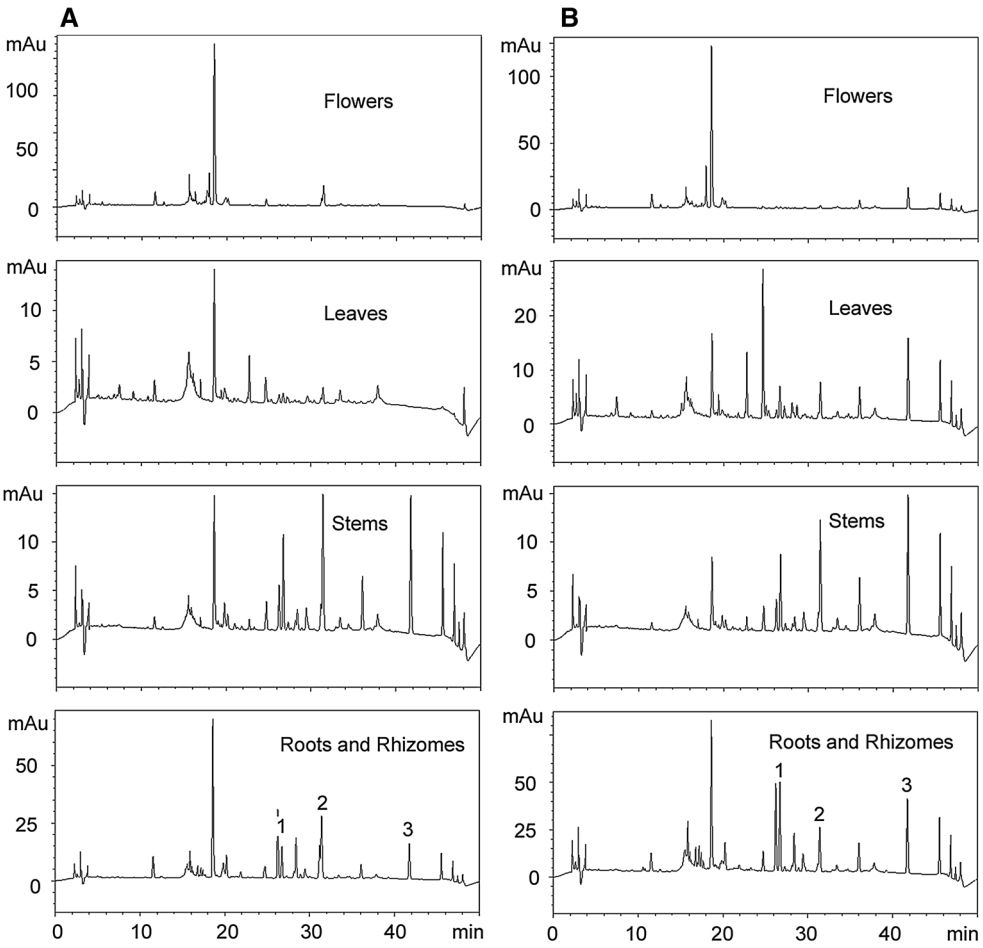

Fig. 8 HPLC chromatograms detected at 254 nm: diploid plant (**a**) and tetraploid plant (**b**)

Table 4 Alkamide contents (total area of identified peaks/g) in different parts of diploid and tetraploid plants

Plant ploidy	Flowers	Leaves	Stems	Roots and rhizomes
Diploid	1,298,465 ± 105,478a	1,971,495 ± 173,256b	1,594,510 ± 115,857a	6,494,510 ± 478,532b
Tetraploid	1,552,575 ± 120,423a	2,390,240 ± 193,571a	1,875,780 ± 145,793a	8,025,265 ± 654,723a

The values in each vertical column that are followed by different letters are significantly different

Table 5 Activities of PAL and C4H in diploid and tetraploid plants

Plant ploidy	Leaves		Roots and rhizomes	
	PAL (U h^{-1}/g FW)	C4H (U min^{-1}/g FW)	PAL (U h^{-1}/g FW)	C4H (U min^{-1}/g FW)
Diploid	155.89 ± 12.97b	6.48 ± 0.52a	222.61 ± 15.34a	8.14 ± 0.78a
Tetraploid	187.51 ± 14.33a	7.62 ± 0.65a	248.05 ± 17.52a	9.25 ± 1.52a

The values in each vertical column that are followed by different letters are significantly different

is ammonolyzed to cinnamic acid by the action of PAL, and cinnamic acid is hydroxylated to p-coumaric acid by the action of C4H and further hydroxylated to caffeic acid (Hahlbrock and Scheel 1989). Therefore, the impact of the ploidy level on the caffeic acid derivative biosynthesis was studied through the determination of the activities of PAL and C4H. The results are listed in Table 5.

The activities of PAL and C4H in tetraploid plants were higher than those in diploid plants, which indicates that the tetraploid plants exhibit a higher degree of secondary metabolite biosynthesis at the proteomic level.

Discussion

The FTIR spectra and the second derivative spectra of the flowers, stems, leaves, and roots and rhizomes of 2-year-old diploid and tetraploid plants showed that the chemical profiles of diploid and tetraploid plants are similar. The HPLC determination of caffeic acid derivatives and alkamides in the diploid and tetraploid plants showed that the tetraploid plants have higher contents of these compounds. Moreover, the total fresh root weight and total dry root weight of tetraploid plants were found to be 39.32 and 40.48 % higher than those of diploid plants, respectively. Therefore, the induction of polyploidy may be a valid strategy to achieve a higher yield of biomass and bioactive compounds in *E. purpurea*. Tetraploid *E. purpurea* would thus exhibit higher medicinal value and has a good application prospect.

Polyploidy is frequently accompanied by conspicuous changes in morphology and metabolite production (Lavania 2005; Kaensaksiri et al. 2011; Sun et al. 2011; Van Laere et al. 2011; Dehghan et al. 2012). Similarly, our observations also revealed that tetraploid plants have higher contents of caffeic acid derivatives and alkamides than diploid *E purpurea* plants. However, the molecular mechanisms through which polyploidy affects secondary metabolites remain obscure. It is known that polyploidy is not simply a chromosome doubling and is always accompanied by changes in the genome structure and gene expression model (Comai 2000; Wendel 2000; Kashkush et al. 2002; Adams et al. 2004; Adams and Wendel 2005; Chen and Ni 2006). Furthermore, genomic plasticity has downstream effects on the transcriptome, proteome, and metabolome that can generate phenotypic variation in polyploids that exceed that found in the parents (Leitch and Leitch 2008).

Current evidence reveals that increased PAL activity is accompanied with increased concentrations of cichoric acid, caftaric acid, and chlorogenic acid in *E. purpurea* hairy roots (Abashi et al. 2012). We found that the PAL and C4H activities in tetraploid plants were stronger than those in diploid plants. The enhancement of PAL and C4H activities was accompanied with an increase in the cichoric acid content, which indicates that the induction of polyploidy in *E. purpurea* resulted in higher PAL and C4H expression and promoted that biosynthesis of cichoric acid. The correlation between the ploidy level and a certain metabolite is not clear at present (Buggs et al. 2009). However, after chromosome doubling, an increased number of genes may lead to an increased concentration and activity of some enzymes (Caruso et al. 2011). The enhancement of PAL and C4H activities and cichoric acid content might be caused by the upregulation of the corresponding genes. In addition, the genes that regulate the biosynthesis of cichoric acid in tetraploid plants may be dose-dependent (Hegarty and Hiscock 2008). In the future, the study of the variation in the mRNA levels of the genes encoding PAL and C4H in *E. purpurea* varieties with different ploidy levels will provide additional evidence to elucidate the regulatory mechanism through which the ploidy level impacts the contents of metabolites in *E. purpurea*.

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