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ORIGINAL PAPER

In vitro induction of polyploidy in *Centella asiatica* (L.) Urban

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Abstract Mutational breeding was conducted using in vitro-grown shoot-tips of *Centella asiatica* (L.) Urban treated with colchicine (0.025–0.400% for 12–36 h) to induce polyploidy. Treated shoot-tips were grown on Murashige and Skoog (MS) medium supplemented with 4.54 μM thidiazuron (TDZ), and regenerated plantlets were acclimatized and transferred to soil. Two months following transfer to ex vitro conditions, ploidy levels of regenerated plants were determined by flow cytometry and by determining chromosome counts. Treating shoot-tips with colchicine concentrations ranging from 0.050–0.200% for 12–24 h promoted induction of tetraploids. Morphological and growth characteristics and the triterpenoid contents of the polyploids were also measured. Tetraploid plants demonstrated significantly longer stomata and a higher stomatal index compared to those of the diploid control plants. Furthermore, a positive trend in both biomass and triterpenoid production was obtained with the tetraploid and mixoploid plants of *C. asiatica*.

Keywords Apiaceae · *Centella asiatica* · Chromosome · Medicinal plant · Mutational breeding · Triterpenoids

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Introduction

Centella asiatica (L.) Urban (Apiaceae), a tropical herb, grows in moist areas from sea level up to 2,500 m in altitude (Hedge and Lamond 1992). The plant is commonly used as a vegetable, and it is also considered to be a medicinal herb in tropical Asian countries. *C. asiatica* has been clinically proven to be effective for the treatment of wounds (Kartnig 1996) and venous hypertensive microangiopathy (Cesarone et al. 2001). Furthermore, *C. asiatica* has demonstrated anxiolytic activity (Bradwejn et al. 2000) and has been shown to attenuate age-related cognitive decline and mood disorders in the healthy elderly (Wattanathorn et al. 2008). In Thailand, the Ministry of Public Health has recommended *C. asiatica* for wound healing in the Essential Drug List of Herbal Medicinal Products. Therefore, the demand for high-quality *C. asiatica* raw material has increased.

Polyploidy is the heritable condition in which cells contain more than two complete sets of chromosomes. In plants, it was estimated that polyploidy has an occurrence of between 30 and 70% (Wolfe 2001). Polyploidy has a common role in speciation, and it has been estimated that approximately 2–4% of speciation events in angiosperms are associated with polyploidy (Otto and Whitton 2000). Induction of polyploid plants has been of considerable interest for researchers and has been used for obtaining new plant characteristics (Cheng and Korban 2011). In vitro chromosome doubling can be induced by several antimitotic agents (Dhooghe et al. 2011). The most commonly used are colchicines such as in *Lagerstroemia indica* (Zhang et al. 2010), *Paulownia tomentosa* (Tang et al. 2010), oryzalin in *Smallanthus sonchifolius* (Viehmannová et al. 2009) and trifluralin in *Ranunculus* (Dhooghe et al. 2009). A number of published papers have reported that artificial tetraploidy in medicinal plants increases the

amount of biomass or phytochemicals, such as *Artemisia annua* (Wallaart et al. 1999), *Salvia miltiorrhiza* (Gao et al. 1996), *Papaver somniferum* (Mishra et al. 2010), and *Scutellaria baicalensis* (Gao et al. 2002).

Chulalaksananukul and Chimnoi (1999) have reported the induction of polyploidy in *C. asiatica* using 2% colchicine applied via the cotton ball method. Nevertheless, the chromosomal characteristics of those plants were not well defined, as the polyploidy was identified by the phenotypes and the number of guard cell chloroplasts. The present investigation was aimed at the induction of polyploidy in *C. asiatica* for the selection of elite lines to be used for plant material production in the herbal industry. In this study, an appropriate protocol for in vitro tetraploid induction in *C. asiatica* was established. Furthermore, the morphological and chromosomal characteristics, the biomass, and the secondary metabolite accumulation of *C. asiatica* at different ploidy levels were assessed.

Materials and methods

Plant multiplication

A *Centella asiatica* mother plant was obtained from Siriruckhachati Medicinal Plant Garden, Mahidol University, Thailand. The plant was propagated under greenhouse conditions. Shoot-tips from the stolons (1.5 cm long) were treated with a mixture of 2 gl^{-1} Funguran® and 2 gl^{-1} Orthocite® for 30 min, soaked with 70% ethanol for 1 min, sterilized with 3% sodium hypochlorite solution containing 0.1% Tween 80® for 10 min, and then rinsed 3 times with sterilized distilled water. Each shoot-tip explant was cultured in a 4-oz (113.6-ml) bottle containing 20 ml semi-solid MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose and 5.5 gl^{-1} Agargel®, pH 5.8. The explants were cultured at 25°C under a 16 h/8 h (light/dark) photoperiod with a light intensity of 27 μmol m^{-2} s^{-1}, provided by cool-white fluorescent tubes. The established cultures of *C. asiatica* were pre-treated in a 125-ml flask containing 50 ml liquid MS supplemented with 4.54 μM thidiazuron for 15 days on a shaker at 100*g*; the cultures were then transferred to semi-solid MS without plant growth regulator (PGR) for 5 weeks. Subsequently, clusters of shoots were cut, transferred to new semi-solid MS medium without PGR, and incubated for an additional 5 weeks (Kaensaksiri et al. 2011). Plants were transferred to fresh culture medium, and used for induction of polyploids.

Polyploid induction

In vitro-grown shoot-tips of *C. asiatica* were soaked in colchicine solution, at different concentrations ranging between 0.025 and 0.400% for 12–36 h, and then washed three times with sterilized distilled water. Sterilized distilled water was used as a control. Fifty-four explants were used in each treatment.

For shoot induction, the treated explants were incubated in liquid MS supplemented with thidiazuron, as described above. All regenerated shoots were designed as the first vegetative generation of a mutagenized plant (M_1V_1). The shoots of the M_1V_1 plantlets were regenerated using the same protocol for an additional 2 passages to obtain M_1V_2 and then M_1V_3 plants.

Flow cytometry analysis

The in vitro control and regenerated plants (M_1V_1 and M_1V_3) were analyzed for ploidy level. Cells were prepared using a method that was a modification of the protocol described by Pfosser et al. (1995). Nuclei were isolated from a mature leaf (0.5 × 1 cm^2) by chopping with a sharp razor blade in 500 μl Tris buffer containing 0.2 M Tris, 4 mM $MgCl_2 \cdot 6H_2O$, and 0.5% (v/v) Triton X-100, pH 7.5. The suspension was filtered through a 40-μm cell strainer and then washed with 500 μl Tris buffer. The nuclei were treated with RNase, stained with propidium iodide, and incubated in the dark at 4°C for 1–2 h before the analysis with a flow cytometer (BD FACSCanto™ System). The stained nuclei were analyzed at a concentration of 5,000 nuclei per sample. Leaves of *C. asiatica* ($2n = 2x = 18$) were used as the internal standard. For each tetraploid sample, two replicates were performed.

Chromosome count

The ploidy level of the mother plant and in vitro polyploid plants, assessed by flow cytometry, was confirmed with chromosome count. Root tips were pretreated with ice water (4°C) for 27 h and then fixed in Carnoy's solution (glacial acetic acid: 95% ethanol, 1:3) for 1 day. After the fixative was washed out with water, the fixed roots were hydrolyzed in 1 N HCl at 60°C for 10 min. The samples were prepared using the Feulgen squash method, by soaking the tissues in Schiff's reagent for 10 min and squashing the samples with 2% aceto-orcein dye on the microscopic slide. The chromosome numbers were observed under a light microscope with ×1,000 magnification.

Stomatal characteristics

The ex vitro leaves of M_1V_3 were fixed in Carnoy's solution. After 1 day, the fixative was washed out with water. A leaf was then placed on a microscope slide and dried with 2% aceto-orcein. The lower surface was

Table 1 The effect of concentration and duration of colchicine treatments on plant survival and ploidy levels in *C. asiatica* (M_1V_1)

Colchicine concentration (%)	Duration (h)	Number of plants	Surviving plants after 3 months	Ploidy levels of treated plants		
				Diploid (2x)	Tetraploid (4x)	Mixoploid (2x + 4x)
0	12	54	54	54	0	0
	24	54	51	51	0	0
	36	54	53	53	0	0
0.025	12	54	3	3	0	0
	24	54	4	2	0	2
	36	54	1	1	0	0
0.050	12	54	3	2	1	0
	24	54	3	1	0	2
	36	54	1	0	0	1
0.100	12	54	2	0	2	0
	24	54	2	0	1	1
	36	54	1	1	0	0
0.200	12	54	1	1	0	0
	24	54	1	0	1	0
	36	54	0	0	0	0
0.400	12	54	0	0	0	0
	24	54	1	1	0	0
	36	54	0	0	0	0

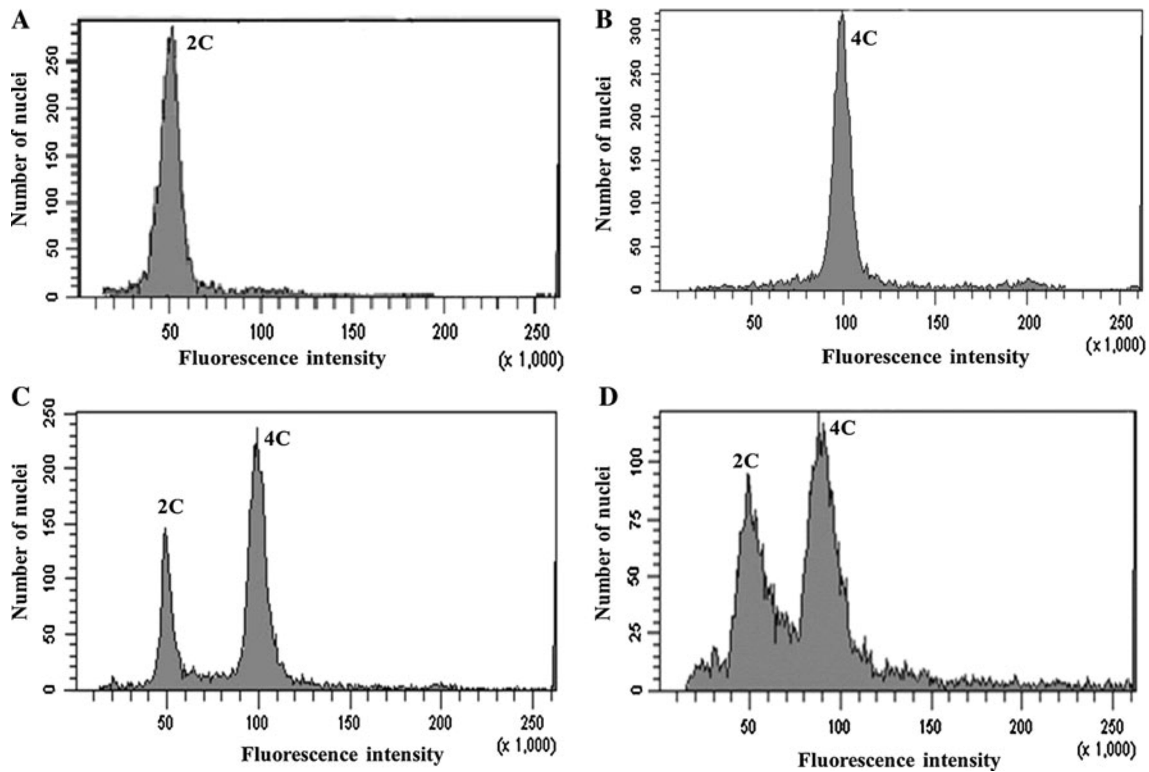

Fig. 1 Flow cytometry histogram of *C. asiatica* (M_1V_3) grown under greenhouse conditions for 2 months. **a** Histogram of a diploid control plant, **b** histogram of a tetraploid plant, **c** histogram of a mixoploid plant (2x + 4x), **d** histogram of a mixture of a diploid control plant (internal standard) and a tetraploid plant

observed under the light microscope, and then the stomatal index calculation was performed according to the formula: Stomatal Index (%) = [$S \times 100/(E + S)$], where S is the number of stomata and E is the number of epidermal cells (Mishra 1997).

Cultivation and harvest

In vitro M_1V_3 polyploid and control diploid plants were hardened at room temperature (30 ± 2°C) for 1 week, and then the roots were washed with tap water. The plantlets were transplanted to plastic trays containing sand and rice shell ash (1:1) and grown in a mist tent for 3 weeks. The plantlets were then transferred and grown under greenhouse conditions for 1 week. After that, each surviving plant was transferred to a 6-inch (15.25-cm) plastic pot containing soil for 1 month. The plants were grown from September to November 2009, at a temperature of 23–33°C. The leaves of the plant (M_1V_3) were collected at 2 months after transplantation, dried in a hot-air oven at 50°C for 48 h and ground into powder with a Retsch® mill.

HPLC conditions

The leaf powder was extracted by sonication in 80% methanol and then analyzed using a LiChroCART® column (LiChrospher® 100 RP-18 250 mm × 4 mm I.D.; particle size 5 µm) with a Perkin-Elmer HPLC system. The mobile phase, consisting of acetonitrile (A) and 0.05% phosphoric acid in water (B), was modified from the protocol described by Rafamantanana et al. (2009). The gradients of solvent A were varied as follows: 20–35% A (15 min), 35–65% A (10 min), 65–80% A (5 min), 80–20% A (5 min), and 20% A (5 min) with a flow rate of 0.8 ml min^{-1}. Asiaticoside (Fluka), asiatic acid (Sigma), madecassoside (Extrasynthese), and madecassic acid (Extrasynthese) were used as external standards and their absorbances were measured at 206 nm.

Statistical analysis

The analysis of the effect of the ploidy level on the length of the stomata, width of the stomata, stomatal index, stolons per plant, inflorescences per node, length of the

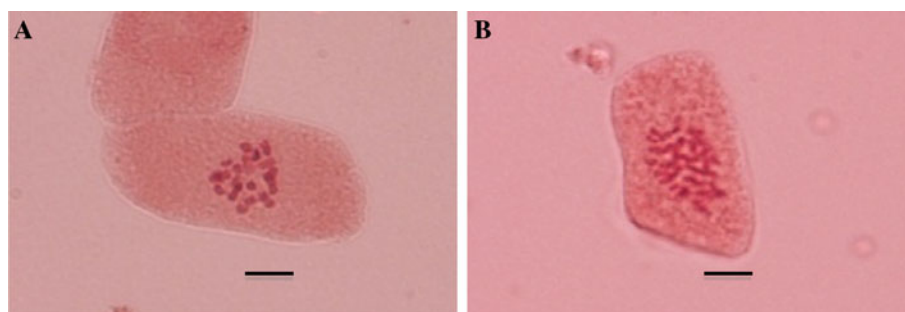

Fig. 2 Chromosome numbers of *C. asiatica* (M_1V_3). **a** Chromosome number of diploid control plant ($2n = 2x = 18$), **b** chromosome number of tetraploid plant ($2n = 4x = 36$). *Bars* 5 µm

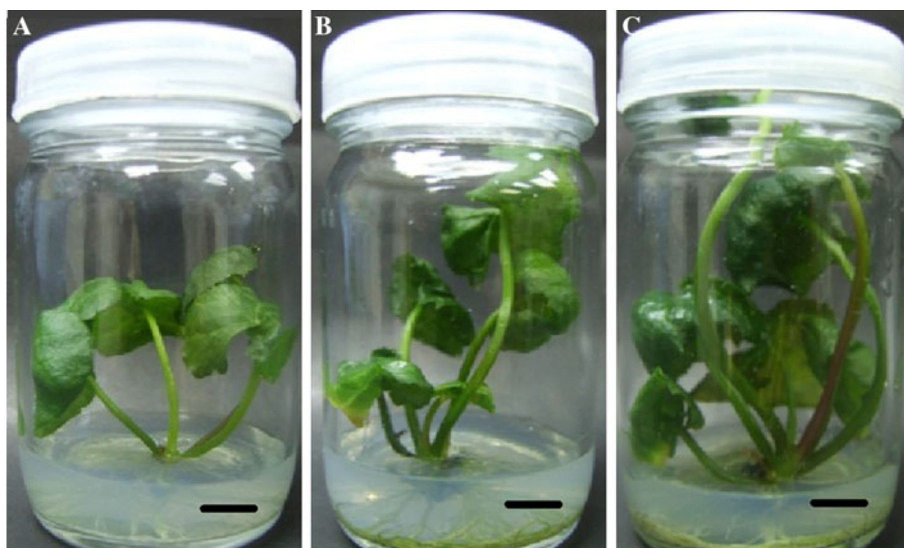

Fig. 3 In vitro *C. asiatica* (M_1V_3) aged 2 months. **a** Diploid control plant, **b** tetraploid plant, **c** mixoploid plant. *Bars* 1 cm

petiole, width of the leaf, length of the leaf, leaves per plant, fresh weight, dry weight, and triterpene content was managed as a completely randomized design (CRD), with 3 replications and analysis with one-way ANOVA. The means were compared with Duncan's Multiple Range Test (DMRT) at a 95% confidence level.

Results and discussion

Polyploid induction

At the 3rd month of growth following the colchicine treatment, all of the explants treated with colchicine demonstrated lower survival rates than those of the control (Table 1). The ploidy level of the surviving plants was determined by flow cytometry and chromosome count recommended by Ochatt et al. (2011). The peak of a diploid control plant (2x) was set at channel 50 (Fig. 1a). The peak of tetraploid plants (4x) was expected at channel 100 (Fig. 1b), and the peaks of mixoploid plants (2x + 4x) were expected at both channels 50 and 100 (Fig. 1c). Figure 1d demonstrates the confirmed peaks of a mixture of a tetraploid with a diploid control plant (internal standard). The ploidy level of the polyploid plants was confirmed by the determination of the chromosome number. The diploid control plants had a chromosome number of $2n = 2x = 18$ (Fig. 2a), whereas the tetraploid plants had a chromosome number of $2n = 4x = 36$ (Fig. 2b).

In M_1V_1 generation, the tetraploid plants (4x) were obtained by treatment with 0.050–0.200% colchicine solutions, with immersion times of 12–24 h. Mixoploid plants (2x + 4x) were obtained using 0.025–0.100% colchicine, with immersion times of 24–36 h. These results indicated that the optimum tetraploid induction was performed with a higher concentration of colchicine and a shorter immersion time than those of the mixoploid plants (Table 1). The in vitro diploid, tetraploid, and mixoploid plants (M_1V_1) were micropropagated to obtain M_1V_2 and M_1V_3 plants. A tetraploid M_1V_1 plant was in vitro regenerated to obtain 8 M_1V_2 plants and then 60 M_1V_3 plants. Thirty-nine M_1V_3 plants were randomly selected for growing under greenhouse conditions for 2 months. Confirming by flow cytometry analysis, we found that the plants were 3 tetraploids, 9 diploids and 27 mixoploids. This may be related to a chimera effect. In mixoploid plant, 1 M_1V_1 plant was in vitro multiplied to get 17 M_1V_2 plants, of these were 5 mixoploid and 12 diploid plants. Fifty M_1V_3 plants were obtained from those 5 mixoploid plants. The ploidy level of the regenerated plants (M_1V_3) was confirmed by flow cytometry, 2 months after transfer to the greenhouse. Twenty-five of 50 plants were randomly analyzed by flow cytometry; 24 plants were still mixoploids. Figure 3 shows in vitro M_1V_3 plants at different ploidy levels.

Morphological characteristics and triterpenoid production of *C. asiatica* at different ploidy levels

The stomatal characteristics of *C. asiatica* plants at different ploidy levels are shown in Fig. 4. The results demonstrated that the average length of the stomata of tetraploid *C. asiatica* was significantly longer than that of the diploid control plants ($P = 0.006$), whereas the width of the stomata was not significantly different. The stomatal

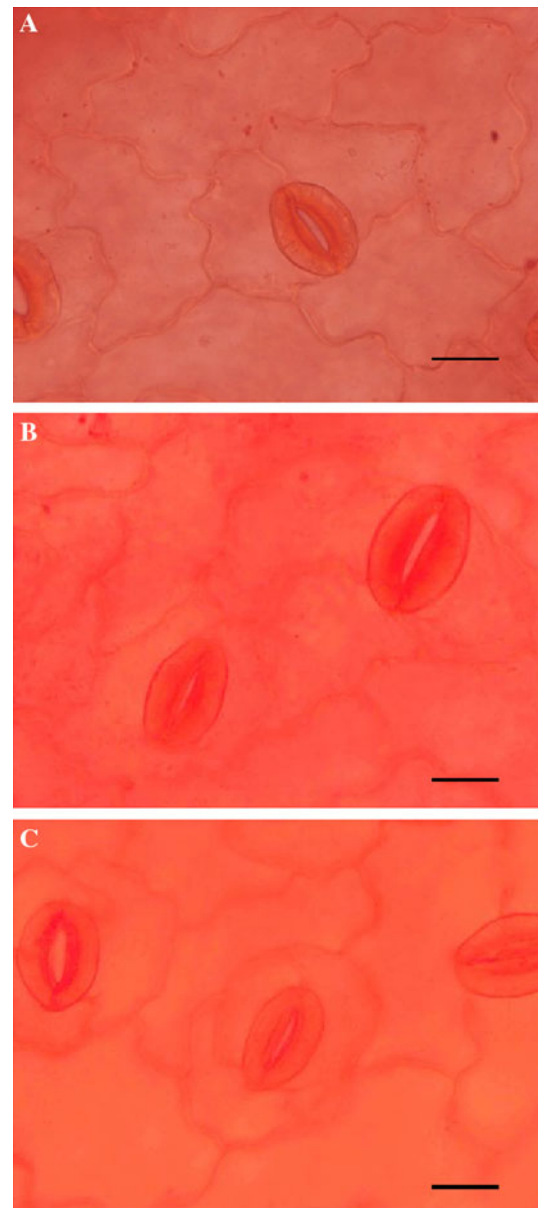

Fig. 4 Stomata of lower epidermis of *C. asiatica* (M_1V_3). **a** Diploid control plant, **b** tetraploid plant, **c** mixoploid plant. *Bars* 20 µm

Table 2 Effect of ploidy level on characteristics and triterpenoid contents of *C. asiatica* (M_1V_3)

Characteristics[a]	Diploid	Tetraploid	Mixoploid	*P* value
Length of stomata (µm)	30.14 ± 0.98 b	37.63 ± 1.37 a	35.42 ± 0.63 a	0.006
Width of stomata (µm)	22.92 ± 1.67 a	23.89 ± 0.28 a	22.50 ± 0.48 a	0.629
Stomatal index	22.16 ± 0.77 b	26.83 ± 0.70 a	23.48 ± 0.10 b	0.004
Stolons per plant	4.00 ± 0.00 a	2.00 ± 0.58 b	3.00 ± 0.00 ab	0.016
Inflorescences per node	2.00 ± 0.19 b	3.00 ± 0.38 ab	3.90 ± 0.49 a	0.033
Length of petiole (cm)	5.41 ± 0.63 a	6.86 ± 1.10 a	6.49 ± 0.57 a	0.469
Width of leaf (cm)	4.80 ± 0.29 a	5.17 ± 0.32 a	4.94 ± 0.24 a	0.665
Length of leaf (cm)	4.06 ± 0.24 a	4.33 ± 0.33 a	4.52 ± 0.16 a	0.500
Leaves per plant	7.67 ± 0.33 a	6.00 ± 0.58 b	5.67 ± 0.33 b	0.035
Fresh weight (g/plant)	2.02 ± 0.21 a	3.57 ± 0.68 a	2.34 ± 0.42 a	0.126
Dry weight (g/plant)	0.38 ± 0.02 a	0.51 ± 0.09 a	0.43 ± 0.03 a	0.344
Madecassoside (%w/w)	4.81 ± 0.75 a	3.04 ± 0.29 a	3.32 ± 1.09 a	0.302
Asiaticoside (%w/w)	2.94 ± 1.71 a	5.29 ± 0.68 a	5.24 ± 0.30 a	0.286
Madecassic acid (%w/w)	ND	0.35 ± 0.02 a	0.29 ± 0.15 a	0.056
Asiatic acid (%w/w)	0.20 ± 0.02 a	0.15 ± 0.15 a	0.22 ± 0.03 a	0.864
Total triterpenes (%w/w)	7.95 ± 2.45 a	8.83 ± 0.71 a	9.07 ± 1.22 a	0.880

Means within each row with different letters are significantly different at $P = 0.05$ (DMRT)

ND Not detectable

[a] Values for all parameters correspond to mean ± SE of three samples

Fig. 5 Plant morphology of *C. asiatica* (M1V3). **a** Diploid control plant, **b** tetraploid plant, **c** mixoploid plant. *Bar* 5 cm

index ($P = 0.004$) increased significantly in the tetraploid plants, as compared with the diploid plants (Table 2). Our results confirmed that the stomata of polyploid plants are larger than those of diploid plants, as was previously reported for *C. asiatica* (Chulalaksananukul and Chimnoi 1999), *Chrysanthemum cineraiifolium* (Liu and Gao 2007), *Lagerstroemia indica* (Zhang et al. 2010), *Morus alba* (Chakraborti et al. 1998), *Brachiaria ruziziensis* (Ishigaki et al. 2009), *Bixa orellana* (Carvalho et al. 2005), and *Salvia coccinea* (Kobayashi et al. 2008). Furthermore, we found that the number of stomata, as indicated by the stomatal index, of *C. asiatica* tetraploid plants was higher than that of the diploid plants, which has also been reported in *Lagerstroemia indica* (Zhang et al. 2010) and *Bixa orellana* (Carvalho et al. 2005). Thus, we conclude that the stomatal size and index could be used for the identification of polyploids.

Concerning the growth and development of the plants, the tetraploid plants displayed a significantly decreased number of leaves per plant ($P = 0.035$) and exhibited a decrease in stolonification ($P = 0.016$), though they tended to show an increase in the number of inflorescences with a normal appearance ($P = 0.033$) (Table 2). Figure 5 shows diploid, tetraploid, and mixoploid plants of *C. asiatica*, 2 months after transplanting and grown under greenhouse conditions.

The fresh weight of the *C. asiatica* tetraploid plants was increased by more than 77% versus that of the diploid plants, whereas the dry weight increased by approximately 34% (Table 2). Nevertheless, biomass did not significantly increase in induced polyploidy plants. In the induced tetraploid plants, there was both an increase in biomass, such as has been reported for *Scutellaria baicalensis* (Gao et al. 2002), *Salvia miltiorrhiza* (Gao et al. 1996), and *Papaver*

somniferum (Mishra et al. 2010), and a decrease in biomass, similar to that observed in *Artemisia annua* (Wallaart et al. 1999).

The major chemical constituents of the greenhouse-grown *C. asiatica* plants (of all ploidy levels) were determined to be triterpene glycosides (asiaticoside and madecassoside), as was found with *C. asiatica* from Madagascar (Rafamantanana et al. 2009; Randriamampionona et al. 2007) and from in vitro-cultured *C. asiatica* (James et al. 2008). The tetraploid plants showed a non-significant increase of 11% in total triterpenes over that of the diploid plants; such increases have been reported previously in artificial tetraploid plants, such as *Papaver somniferum*, which had an increase of 25–50% in the morphine content (Mishra et al. 2010), and *Artemisia annua*, which showed an increased artemisinin content of 38% (Wallaart et al. 1999). However, Gao et al. (1996, 2002) have reported that the amount of active constituents also depended on the plant genotype; 2 lines (from 10 tetraploid lines) of *Salvia miltiorrhiza* showed an increase of 8.90–78.74% in total tanshinones and cryptotanshinone, whereas the other lines showed a decrease (Gao et al. 1996). In *Scutellaria baicalensis,* one tetraploid line exhibited an increase in baicalin of 4.6%, whereas an additional 19 lines showed reduced baicalin production (Gao et al. 2002).

A mixoploid is one type of a chimera that consists of diploid cells (2x) and tetraploid cells (4x). However, the plants are difficult to identify; therefore, in this study, we used a flow cytometry technique to identify the ploidy level. *C. asiatica* mixoploid plants showed an increase in biomass and triterpenoid contents that resembled that of the tetraploid. Nevertheless, the ploidy levels of the plants regenerated from a mixoploid plant were unstable (data not shown), which was not appropriate for mass production purposes.

In conclusion, we have demonstrated the induction of tetraploid plants of *C. asiatica* that exhibited positive trends in both biomass and triterpenoid production. Further elite tetraploid lines will be selected and used for additional field experimentation.

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