Breeding Science 66: 169–174 (2016)
doi:10.1270/jsbbs.66.169

Research Paper

In vitro induction and morphological characteristics of octoploid plants in *Pogostemon cablin*

Han-Jing Yan, Yang Xiong, Hong-Yi Zhang and Meng-Ling He*

School of Traditional Chinese Medicine, Guangdong Pharmaceutical University, Guangzhou Guangdong, 510006 China

This paper describes an efficient colchicine-mediated technique for *in vitro* induction of octoploids in *Pogostemon cablin* and its confirmation by flow cytometry and chromosome numbers. The highest octoploid induction ratio was obtained by 0.05% colchicine treatment for 72 h. The chromosome number of octoploid seedlings was $2n = 8x = 128$. Colchicine-induced tetraploids and octoploids planted in soil remained stable after 6 months. There were 31 lines of octoploid plants obtained. The leaf characteristics of *P. cablin* tetraploids and octoploids were compared. The larger leaves and stomata of transplants can be used to identify putative octoploids in *P. cablin*. Most octoploid lines exhibited higher patchoulic alcohol contents than the controls after 6 months of cultivation. Our results demonstrated that polyploidy induction can be beneficial in improving the medicinal value of *P. cablin*.

Key Words: *Pogostemon cablin* (Blanco) Benth., colchicine, leaf characteristic, patchoulic alcohol, octoploidy.

Introduction

Pogostemon cablin, of the genus *Pogostemon*, is a perennial herbal plant native to the Philippines. It is a traditional Chinese medicinal material commonly used in removing dampness, relieving summer-heat, exterior syndrome, stopping vomiting and stimulating the appetite. Recent studies showed that *P. cablin* has an *in vitro* antivirus effect (Kiyohara *et al.* 2012, Peng *et al.* 2011, Wang *et al.* 2011). During the Song dynasty, it was introduced from countries in Southeast Asia such as the Philippines and Indonesia to southern China. In China, *P. cablin* is generally propagated by cutting propagation because it does not bloom in southern China. This mode of reproduction leads to weaker resistance of progeny, and a decline in yield and quality. As the plant does not flower in southern China, and only reproduces asexually through stem cutting, it is very difficult to breed new cultivars using traditional methods. Consequently, it is not only necessary to develop new technologies to create germplasm resources, but also breed new cultivars with rapid growth and stable (and higher) content of patchouli oils.

In vitro multiplication of *P. cablin* has been performed using explants of stems (He *et al.* 2009), roots (Xiao *et al.* 2001), leaves (Du *et al.* 2002), callus (Zhang *et al.* 1994) and protoplasts (Mo *et al.* 2012). However, reports about *in vitro* polyploid induction in *P. cablin* are limited. Wu (Wu and Li 2013) induced tetraploids ($2n = 4x = 56$) by immersing shoot tips in 0.02% colchicine solution for 2 h before culture. This chromosome number differed from that found by Lavania (1984), Tyagi and Bahl (1990) and Chen *et al.* (2009) of $2n = 32$ or $2n = 64$. The basic chromosome number is $x = 16$ or 17 in *Pogostemon* (Cherian and Kuriachan 1993). From preliminary experiment results, we agree with the latter opinion that $2n = 2x = 32$ or $2n = 4x = 64$, and $2n = 4x = 64$ is predominant in China (Xiong *et al.* 2013).

Polyploid cells contain more than two complete sets of chromosomes and are heritable. Polyploidy is estimated to have an occurrence rate in the range of 30–70% in plants (Wolfe 2001). Induction of polyploid plants has been of considerable interest for researchers (Cheng and Korban 2011). Characteristics such as larger leaves, stems, roots and flowers in polyploid compared to diploid plants can often be obtained (Watrous and Wimber 1988, Wimber *et al.* 1987). Thus, polyploid plants may have increased biomass and yield.

In vitro chromosome doubling can be induced by several antimitotic agents (Dhooghe *et al.* 2011), such as colchicine in *Lagerstroemia indica* (Zhang *et al.* 2010) and *Paulownia tomentosa* (Tang *et al.* 2010); oryzalin in *Dendrobium*, *Epidendrum*, *Odontioda* and *Phalaenopsis* orchids (Miguel and Leonhardt 2011); and trifluralin in *Ranunculus* (Dhooghe *et al.* 2009). There have been a number of reports of artificial tetraploid medicinal plants—such as *Artemisia annua* (Wallaart *et al.* 1999), *Dioscorea zingiberensis* (Huang *et al.* 2008), *Papaver somniferum* (Mishra *et al.*

Communicated by T. Yamamoto
Received April 8, 2015. Accepted October 20, 2015.
*Corresponding author (e-mail: hmldf@126.com)

2010) and *Scutellaria baicalensis* (Gao *et al.* 2002)—with increased biomass or higher content of phytochemicals.

To develop superior varieties of *P. cablin*, we report here a method for generating polyploid plants using colchicine. Octaploid lines of *P. cablin* were obtained and identified by flow cytometry, root-tip chromosome determination and stomatal observations.

Material and Methods

Plant material and in vitro multiplication

A tetraploid *P. cablin* mother plant was obtained from Guangzhou and planted in the medicinal plant garden in Guangdong Pharmaceutical University. For *in vitro* cultures, leaves were treated with 75% ethanol for 5 s, then with 0.1% mercuric chloride for 10–15 min, washed three times (2–3 min each) with sterile water and cultured in shoot multiplication medium: MS medium (Murashige and Skoog 1962) supplemented with 0.2 mg L^{-1} 6-benzylaminopurine and 0.1 mg L^{-1} α-naphthaleneacetic acid for cluster bud multiplication. Cultures were maintained at 25 ± 2°C with 16 h per day photoperiod at a light intensity of 60 μE m^{-2} s^{-1}.

Octoploid induction

Liquid MS medium supplemented with 2% dimethyl sulfoxide and filter-sterilized colchicine (0.05, 0.1 and 0.2% final concentrations) was used for octoploid induction. Cluster buds (3–6 mm) excised from *in vitro* cultures were placed in MS liquid medium containing the respective concentrations of colchicine described above as well as in colchicine-free MS medium and incubated by shaking (100 rpm) at 25°C for 12, 24, 36, 48, 60, 72 and 84 h. A total of 40 explants were used per treatment. Shoot apices were washed three times (2–3 min each) with sterile water and cultured in shoot multiplication medium for plant regeneration.

Flow cytometry analysis of ploidy level

The ploidy level of regenerated plants of *in vitro* mother cultures and colchicine-treated cultures were analyzed by flow cytometry (Becton Dickinson Immunocytometry Systems, California, USA). Cells were prepared using a method modified from Loureiro *et al.* (2007). Nuclei were isolated from mature leaves (0.5 cm × 1 cm) by chopping with a sharp razor blade in 2 mL of Tris-HCl buffer containing 0.2 mol L^{-1} Tris, 4 mmol L^{-1} $MgCl_2$, 2 mmol L^{-1} EDTA-Na_2, 86 mmol L^{-1} NaCl, 10 mmol L^{-1} $Na_2S_2O_5$, 1% PVP-10 and 1% (v/v) Triton X-100 at pH 4.5. The suspension was filtered through a 40-μm cell strainer and then washed with 100 μL of Tris-HCl buffer. The nuclei were treated with RNase, stained with propidium iodide and incubated in darkness at 4°C for 1–2 h. The stained nuclei were diluted to a concentration of 5000 nuclei per sample and analyzed for ploidy level by a flow cytometer.

Chromosome count

The ploidy level of the mother plant and *in vitro* polyploid plants, assessed by flow cytometry, was confirmed by chromosome count. Root tips were pretreated with ice-water mixture for 24 h, and then digested in an enzyme mixture of 4% cellulase and 4% pectinase at 35°C for 30–100 min, washed three times in distilled water and subsequently incubated in distilled water at room temperature for 15 min. The samples were then processed using the Feulgen squash method. The tips were placed on pre-cooled microscope slides and squashed in the presence of the fixative. The slides were heated over an alcohol flame to dry the fixative, stained with fresh 5% Giemsa in Sorensen's buffer at room temperature for 20 min, washed with a fresh stream of water and dried at room temperature. The chromosome numbers were observed under a light microscope (ZEISS Axioplan 2). A minimum of 50 metaphase cells showing well-scattered and contracted chromosomes were counted for each plantlet.

Observation of leaf characteristics

Leaf characteristics were observed in 6-month-old plants. For stomatal measurements, about 0.1 cm^2 of the leaf lower epidermis was used. Four leaves were chosen from each of five tetraploid controls and each of five octoploid plants, and 20 stomata were measured per leaf.

Sample extraction and patchoulic alcohol analysis

Pulverized samples (3 g) of *P. cablin* were weighed accurately and extracted three times with chloroform (50 mL) in an ultrasonic bath for 20 min. The filtrate was combined and evaporated in vacuo and the residue was dissolved in 5 mL of n-hexane. Of the above solution, 1 mL and 0.05 mL of internal standard were added to a 5-mL volumetric flask and diluted to volume with n-hexane. The obtained solution was filtered through a syringe filter (0.45 μm). AT-SE54 capillary column (0.25 mm × 15 mm, 0.25 μm); carrier gas flow rate 1.0 mL min^{-1}; keep 5 min; and injection volume of 1 μL. The content of patchouli alcohol was determined by gas chromatography (GC) method according to the Chinese Pharmacopoeia (China Pharmacopoeia Committee 2015).

Experimental data analysis

Data were analyzed using SPSS ver. 10.0 (SPSS, Chicago, IL). Significant differences among the means were separated using the two-sample *t*-test at $p < 0.05$.

Results

Survival and growth of colchicine-treated buds

The effect of colchicine on growth of the explants was assessed 3 months after treatment (**Table 1**). Both apical and lateral buds were counted. Non-growing brown buds were considered to be dead.

The first visible effect of colchicine was the delayed growth rate of explants. The initiation of bud growth occurred within 3–4 d in untreated explants and 10–15 d in colchicine-treated explants. After a month of growth, all

Table 1. Effect of different concentrations and treatment duration of colchicine on polyploidy induction (mean ± standard error) in *P. cablin* (all data are in percentages)

Colchicine concentration (%)	Duration (exposure) time (h)	Treatment number	Survival number	Variation number	Variation rates (%)	
					Of initial buds	Of survival buds
0.05	12	40	84	14	34.89 ± 1.35	16.36 ± 1.29
	24	40	71	21	52.45 ± 2.43	29.43 ± 0.92
	36	40	68	24	60.32 ± 2.89	33.94 ± 3.01
	48	40	65	24	59.56 ± 2.51	36.92 ± 1.02
	60	40	70	30	75.64 ± 2.78	41.27 ± 1.37
	72	40	61	34	85.65 ± 1.1	54.73 ± 2.13
	84	40	54	21	52.53 ± 2.01	38.89 ± 1.32
0.1	12	40	57	19	47.59 ± 3.92	33.33 ± 3.19
	24	40	42	16	40.32 ± 4.02	38.09 ± 3.89
	36	40	37	19	47.57 ± 4.62	51.35 ± 4.58
	48	40	45	22	55.68 ± 3.85	48.88 ± 3.58
	60	40	49	24	60.56 ± 5.01	48.97 ± 4.97
	72	40	35	20	50.56 ± 4.53	57.14 ± 4.62
	84	40	29	15	37.52 ± 3.59	51.72 ± 3.57
0.2	12	40	45	20	50.35 ± 4.69	43.44 ± 5.09
	24	40	35	17	42.54 ± 4.82	46.57 ± 5.01
	36	40	35	21	52.55 ± 3.78	60.75 ± 3.27
	48	40	31	20	50.29 ± 5.96	65.51 ± 4.91
	60	40	29	13	32.54 ± 4.82	42.82 ± 5.14
	72	40	23	12	30.19 ± 4.27	52.31 ± 4.78
	84	40	21	11	27.45 ± 4.87	52.38 ± 3.69

[a] Induction on MS solid medium, and the seedlings transplanted in soil for 6 months.
[b] The seedlings both from the lateral buds and the cluster buds were in the statistical range.

colchicine-treated explants had significantly shorter shoots than untreated explants(data not shown). Colchicine-treated explants also had significantly fewer lateral buds per explant than untreated explants, and the lateral buds number decreased with increasing colchicine concentrations (**Table 1**).

Flow cytometry analysis and chromosome counts of colchicine-treated explants

All surviving colchicine-treated explants were subjected to flow cytometry to determine their ploidy. The peak of a tetraploid control plant (4*x*) was set at channel 80 (**Fig. 1A**). The peak of the octoploid plants (8*x*) was expected at channel 160 (**Fig. 1B**). The ploidy level of the polyploid plants was confirmed by chromosome count. The tetraploid control plants had a chromosome number of $2n = 4x = 64$ (**Fig. 2A**), whereas the octoploid plants had $2n = 8x = 128$ (**Fig. 2B**). The highest percentage of octoploid induction was 68% and occurred in the 0.05% colchicine treatment for 72 h.

The chromosome counts and flow cytometry indicated that 31 octoploid plantlets were obtained.

Stomatal observation

Table 2 shows the comparison of stomata characteristics between tetraploids and octoploids. The mean stomatal length and width were 41.41 and 29.07 µm, respectively, in 3-month-old octoploids, but only correspondingly 29.15 and 23.00 µm in 3-month-old tetraploids (**Fig. 3**, **Table 2**). The average stomatal frequency in tetraploids was 7.01 mm^{-2} and that in 3-month-old octoploids was 3.48 mm^{-2} (**Table 2**). There was an average of 6.36 chloroplasts per stomata in 3-month-old tetraploids; this number doubled to 14.04 in 3-month-old octoploids (**Table 2**). All stomatal characteristics of 6-month-old tetraploids and octoploids were also significantly different.

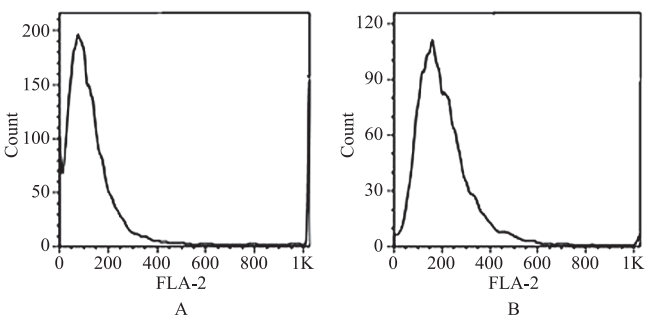

Fig. 1. Flow cytometry histogram of *P. cablin* grown under greenhouse conditions for 6 months. Histogram of a tetraploid control (A) and an octoploid (B) plant.

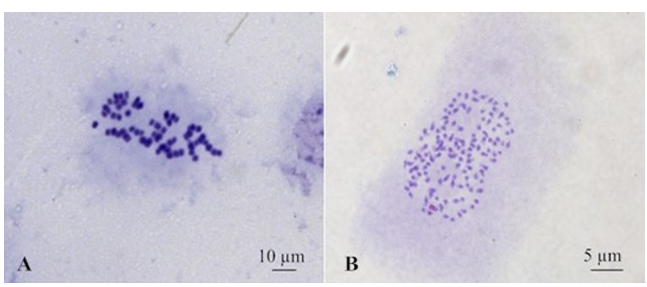

Fig. 2. Chromosome numbers of tetraploid (A) and octoploid (B) *P. cablin*.

Table 2. Stomatal characteristics of tetraploid and octoploid *P. cablin*

Ploidy	Stomatal Length (μm)	Stomatal Width (μm)	Stomatal density (no./mm^2)	chloroplasts number (no./stoma)
Tetraploid (3 month)	29.15 ± 0.48a	23.00 ± 0.29a	7.01 ± 0.2a	6.36 ± 0.44a
Octoploid (3 month)	41.41 ± 0.68b	29.07 ± 0.49b	3.48 ± 0.16b	14.04 ± 0.45b
Tetraploid (6 month)	28.33 ± 3.1a	21.38 ± 2.43a	23.5 ± 4.08c	6.05 ± 1.19a
Octoploid (6 month)	40.95 ± 3.38b	27.82 ± 2.05b	10.9 ± 2.18b	12.3 ± 2.3b

[a] Values within the same column followed by different lower-case letters are significantly different according to two-sample *t*-test ($p < 0.05$).

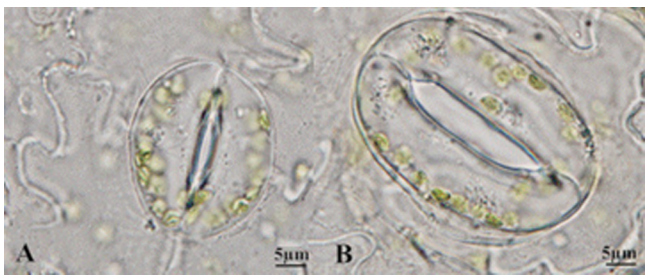

Fig. 3. Stomata characteristics of tetraploid (A) and octoploid (B) *P. cablin*.

These observations are consistent with the results of some studies showing that stomatal index decreased and stomata length and width increased with increasing ploidy (Mishra 1997). The differences in stomatal characteristics between tetraploid and octoploid plantlets were significantly different according to two-sample *t*-test ($p < 0.05$).

Morphological differences between tetraploid and octoploid P. cablin

The morphological features of polyploid plants were evaluated to determine whether they could be used to identify putative octoploids. In most cases, the leaves of octoploid plants appeared normal in shape compared with those of tetraploid plants, but octoploid plants with thicker stems were taller and stronger than tetraloid plants. The length and width of 30-d-old tetraploid and octoploid *in vitro* leaves were not significantly different (data not shown), but those of 3- and 6-month-old plants differed significantly (**Table 3**, **Figs. 4**, **5**). The average leaf length was 6.55 and 7.23 cm in tetraploid and octoploid plants, respectively; and the corresponding leaf widths of 5.08 and 6.84 cm also differed. Thus the surface area was about 1.46 times greater for octoploid than for tetraploid leaves. These characteristics were also significantly different between 6-month-old tetraploid and octoploids, expect for leaf length.

Content of patchoulic alcohol

The content of effective compounds is very important for medicinal plants. In order to evaluate the content of the effective constituent of octoploid *P. cablin*, herb samples of each octoploid plant were extracted and analyzed by GC.

The content of patchoulic alcohol in each octoploid line is shown in **Table 4**. Only two lines, 26 and 27, had a lower content of patchoulic alcohol than the control ($p < 0.05$). The contents of patchoulic alcohol in lines 26, 27 and in the

Table 3. Leaf characteristics (mean ± standard error) of tetraploid and octoploid *P. cablin*

Ploidy	Leaf length (cm)	Leaf width (cm)	Leaf index	Leaf area (cm^2)
Tetraploid (3 month)	6.55 ± 0.21a	5.08 ± 0.14a	1.3 ± 0.03a	24.17 ± 1.10a
Octoploid (3 month)	7.23 ± 0.13b	6.84 ± 0.18b	1.06 ± 0.02b	35.37 ± 1.15b
Tetraploid (6 month)	7.14 ± 0.81b	6.26 ± 0.84b	1.14 ± 0.1a	31.51 ± 4.52c
Octoploid (6 month)	7.3 ± 0.9b	7.12 ± 0.8c	1.03 ± 0.05b	40.23 ± 4.56d

[a] Values within the same column followed by different lower-case letters are significantly different according to two-sample *t*-test ($p < 0.05$).
Leaf index = leaf length/leaf width.

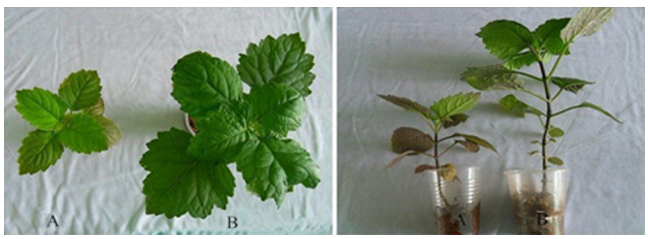

Fig. 4. Plant morphology of tetraploid (A) and octoploid (B) *P. cablin*.

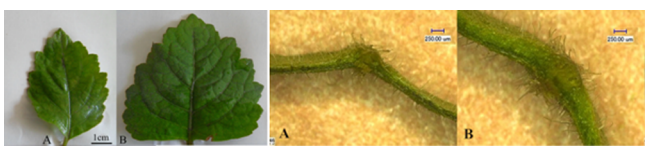

Fig. 5. Leaves of tetraploid (A) and octoploid (B) *P. cablin*.

Table 4. Content of patchoulic alcohol in octoploid *P. cablin*

Strain	Content of patchoulic alcohol (mg/g)	Stain	Content of patchoulic alcohol (mg/g)	Stain	Content of patchoulic alcohol (mg/g)
1	**3.7 ± 0.28***	11	**4.29 ± 0.46***	21	**4.47 ± 0.43***
2	**3.76 ± 0.42***	12	**4.62 ± 0.29***	22	**8.02 ± 0.38****
3	3.2 ± 0.09	13	**4.27 ± 0.29***	23	**5.8 ± 0.43***
4	3.42 ± 0.03	14	**5.66 ± 0.53***	24	**4.19 ± 0.43***
5	**4.51 ± 0.33***	15	**4.55 ± 0.36***	25	**5.44 ± 0.62***
6	**5.37 ± 0.34***	16	3.18 ± 0.31	26	**0.85 ± 0.034***
7	**3.53 ± 0.46***	17	**5.13 ± 0.29***	27	**1.38 ± 0.33***
8	**3.79 ± 0.27***	18	**3.78 ± 0.32***	28	**6.12 ± 0.5****
9	2.6 ± 0.11	19	3.29 ± 0.1	29	**5.79 ± 0.86***
10	3.03 ± 0.27	20	**4.8 ± 0.21***	30	3.12 ± 0.21
31	3.13 ± 0.33				
Original plant	3.08 ± 0.27				

[a] The numbers in boldface are significantly different (*$p < 0.05$, **$p < 0.01$).

control were 0.85, 1.38 and 3.08 mg/g, respectively. Most octoploid plants (21 lines) had a higher patchoulic alcohol content than controls; especially for line 22 with 8.02 mg/g ($p < 0.01$), which was 2.6 times higher than the control.

Discussion

Using the chemical mutagens, colchicine, to induce polyploidy breeding is a frequently used technique, and now there are multiple polyploids of medicinal plants, such as Dioscorea zingiberensis, Centella asiatica, Angelica dahurica, Pinellia ternate, and Astragalus membranaceus.

Morphological identification, chromosome analysis, and determination of stomata are integral methods in polyploid identification; polyploidy plants have been identified by using the above three methods. As a supplementary means, flow cytometry has also been use in polyploidy identification.

The slow growth may be due to the inhibitory effect of colchicine on cell division. Both untreated and colchicine-treated explants grew equally well when sub-cultured, suggesting that colchicine only caused an initial retardation of growth, as observed in *ex vitro* studies (Chen and Gao 2007).

The survival rate decreased with increasing colchicine concentration and treatment time (**Table 1**). This inverse relationship between colchicine concentration and explant survival was expected and in agreement with *ex vitro* (Dwivedi *et al.* 1986, Sikdar and Jolly 1994) and *in vitro* (Chen and Gao 2007, Tang *et al.* 2010) studies in other plants. The concentration and immersion time of colchicine also influenced the variation rate of buds. Although the variation rate of surviving buds was the highest when immersed in 0.2% colchicine for 48 h, the survival rate and the number of lateral buds per explant was lower. So the variation effect was highest when buds were immersed in 0.05% colchicine for 72 h (**Table 1**).

The utility of stomatal size in distinguishing plants with different ploidy levels has been used in other plant types (Chen and Gao 2007, Hamill *et al.* 1992, Sikdar and Jolly 1994, Van Duren *et al.* 1996). We therefore concluded that stomatal observation and chloroplast enumeration represented a rapid and efficient method for screening putative octoploid plants for transplantation into the field.

In our experiments, octoploid plants of *P. cablin* were generated by colchicine. Of these, 21 lines showed higher productivity of patchoulic alcohol, expressed as yield of effective compounds, than the tetraploid mother plant line. These octoploid lines present a promising new tool for breeding programs aimed at increasing production of medicinal compounds from this species.

The leaf size of transplants was a useful parameter for identifying putative octoploids in *P. cablin.* The larger leaves of the octoploids indicated possible higher biomass and greater amounts of desirable compounds.

The major chemical constituents of *P. cablin* (of all ploidy levels) were determined to be patchoulic alcohol. Most octoploid plants showed higher overall productivity of patchoulic alcohol than the controls. Especially line 22 showed a significant increase of 164% in patchoulic alcohol over that of the tetraploid plant; 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 annu*a, which showed an increased artemisinin content of 38% (Wallaart *et al.* 1999). However, Gao *et al.* (2002) have reported that the amount of active constituents also depended on the plant genotype; 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).

Why is the content of patchoulic alcohol higher for octoploids than tetraploids? One reason is due to their higher weight for the increase of octoploids cells, on the other hand, it is speculated that genetic transcription and expression differences in octoploids lead to the increase of secondary metabolites.

In summary, an efficient colchicine-mediated technique for *in vitro* induction of octoploids in *P. cablin* had been established. The larger leaves and stomata of transplants can be used to identify putative octoploids in *P. cablin*. Most octoploid lines exhibited higher patchoulic alcohol contents than the controls after 6 months of cultivation and polyploidy induction could improve the medicinal value of *P. cablin.*

Acknowledgments

This study was financially supported by the Science and Technology Plan Program of Zhongshan, P. R. China (No. 20101H019) and the Science and Technology Plan Program of Guangdong Province, P. R. China (No. [2012]145).

Literature Cited

Chen, L.L. and S.L. Gao (2007) *In vitro* tetraploid induction and generation of tetraploids from mixoploids in *Astragalus membranaceus*. Sci. Hortic. 112: 339–344.

Chen, R.Y., C.B. Chen, G.L. Liang, X.L. Li, L. Chen, Z.G. Wang, X.J. Ma and W.X. Wang (2009) Chromosome Atlas of Major Economic Plants Genome in China: Tomus V. Chromosome Atlas of Medicinal Plants in China. 1st edn. Science Press, Beijing, p. 341.

Cheng, Z.M. and S.S. Korban (2011) *In vitro* ploidy manipulation in the genomics era. Plant Cell Tissue Organ Cult. 104: 281–282.

Cherian, M. and P.I. Kuriachan (1993) Cytotaxonomic studies of the subtribe *Pogostemoninae sensu* Bentham (*Labiatae*) from south India. Cytologia 58: 439–444.

China Pharmacopoeia Committee (2015) Chinese Pharmacopoeia. China Medical Science Press, Beijing, p. 45.

Dhooghe, E., S. Denis, T. Eeckhaut, D. Reheul and M.C Van Labeke (2009) *In vitro* induction of tetraploids in ornamental *Ranunculus*. Euphytica 168: 33–40.

Dhooghe, E., K. Van Laere, T. Eeckhaut, L. Leus and J. Van Huylenbroeck (2011) Mitotic chromosome doubling of plant tissues in vitro. Plant

Cell Tissue Organ Cult. 104: 359–373.
Du, Q., Z.H. Wang and H.H. Xu (2002) Tissue culture and rapid reproduction of *Pogostemon cablin*. Plant Physiol. Comm. 38: 454.
Dwivedi, N.K., A.K. Sikdar, S.B. Dandin, C.R. Sastriy and M.S. Jolly (1986) Induced tetraploidy in mulberry I. Morphological, anatomical and cytological investigations in cultivar *RFS-135*. Cytologia 51: 393–401.
Gao, S.L., B.J. Chen and D.N. Zhu (2002) *In vitro* production and identification of autotetraploids of *Scutellaria baicalensis*. Plant Cell Tissue Organ Cult. 70: 289–293.
Hamill, S.D., M.K. Smith and W.A. Dodd (1992) *In vitro* induction of banana autotetraploids by colchicine treatment of micropropagated diploids. Aust. J. Bot. 40: 887–896.
He, M.J., X.Q. Yang and K. Chen (2009) Study on bud different and callus induction of *Hainan Pachouli in vitro*. J. China Pha. 18: 21–22.
Huang, H.P., S.L. Gao, L.L. Chen and X.K. Jiao (2008) *In vitro* induction and identification of autotetraploids of *Dioscorea zingiberensis*. In Vitro Cell Dev. Biol. Plant 44: 448–455.
Kiyohara, H., C. Ichino, Y. Kawamura, T. Nagai, N. Sato and H. Yamada (2012) Patchouli alcohol: *in vitro* direct anti-influenza virus sesquiterpene in *Pogostemon cablin* Benth. J. Nat. Med. 66: 55–61.
Lavania, U.C. (1984) Chromosome Number Reports LXXXV. Taxon. 33: 756–760.
Loureiro, J., E. Rodriguez, J. Doležel and C. Santos (2007) Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. Ann. Bot. 100: 875–888.
Miguel, T.P. and K.W. Leonhardt (2011) *In vitro* polyploid induction of orchids using oryzalin. Sci. Hortic. 130: 314–319.
Mishra, M.K (1997) Stomatal characteristics at different ploidy levels in Coffea L. Ann. Bot. 80: 689–692.
Mishra, B.K., S. Pathak, A. Sharma, P.K. Trivedi and S. Shukla (2010) Modulated gene expression in newly synthesized auto-tetraploid of *Papaver somniferum* L. S. Afr. J. Bot. 76: 447–452.
Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15: 473–497.
Mo, X.L., Q.Q. Zeng, S.S. Huang, Y.Z. Chen and Z. Yan (2012) The protoplasts isolation and culture of *Pogostemon cablin* cv. *Shipanensis*. Guihaia 32: 669–673.
Peng, S.Z., G. Li, Z. Su, Z.R. Su, F.X. Zhang and X.P. Lai (2011) Effect of different extract parts of *Pogostemon cablin* (Blanco) Benth on *in vivo* anti-influenza virus. Lishizhen Med. Mater. Med. Res. 22: 2578–2579.
Sikdar, A.K. and M.S. Jolly (1994) Induced polyploidy in mulberry (*Morus* spp.): induction of tetraploids. Sericologia 34: 105–116.
Tang, Z.Q., D.L. Chen, Z.J. Song, Y.C. He and D.T. Cai (2010) *In vitro* induction and identification of tetraploid plants of *Paulownia tomentosa*. Plant Cell Tissue Organ Cult. 102: 213–220.
Tyagi, B.R. and J.R. Bahl (1990) A note on new chromosome number in *Pogostemon cablin* Benth. Cell Chromosome Res. 13: 18–20.
Van Duren, M., R. Morpurgo, J. Dolezel and R. Afza (1996) Induction and verification of autotetraploids in diploid banana (*Musa acuminata*) by *in vitro* techniques. Euphytica 88: 25–34.
Wallaart, T.E., N. Pras and W.J. Quax (1999) Seasonal variations of artemisinin and its biosynthetic precursors in tetraploid *Artemisia annua* plants compared with the diploid wild-type. Planta Med. 65: 723–728.
Wang, Y.T., Z.F. Yang, S.S. Zhao, S. Qin, W.D. Guan, Q.D. Huang, Y.S. Zhao, Q. Lin and Z.Y. Mo (2011) Screening of anti-H1N1 active constituents from Radix Isatidis. J. Guangzhou Univ. Tradit. Chin. Med. 28: 419–422.
Watrous, S.B. and D.E. Wimber (1988) Artificial induction of polyploidy in *Paphiopedilum*. Lindleyana. 3: 177–183.
Wimber, D.E., S. Watrous and A.J. Mollahan (1987) Colchicine induced polyploidy in orchids. *In*: Long Beach, CA, Proceedings of the 5th World Orchid Conference, p. 65–69.
Wolfe, K.H. (2001) Yesterday's polyploids and the mystery of diploidization. Nat. Rev. Genet. 2: 333–341.
Wu, Y. and M. Li (2013) Induction of tetraploid plants of *Pogostemon cablin* (Blanco) Benth and its quality evaluation. Phcog. J. 5: 281–285.
Xiao, S.E., H. He and H.H. Xu (2001) Study on the tissue culture and plant regeneration of *Pogostemon cablin*. Chin. Med. Mat. 24: 391–392.
Xiong, Y., M.L. He, F. He, S.M. Huang and H.J. Yan (2013) Chromosome sectioning optimization and chromosome counting of three cultivars of *Pogostemon cablin*. Guangdong Agri. Sci. 40: 121–123, 131.
Zhang, J.M., X.Q. Zheng and X.P. Sun (1994) Plant regeneration from somatic cells of Cablin Patchouli (*Pogostemon cablin* (Blanco) Benth). Chin. J. Trop. Crop 15: 73–77.
Zhang, Q.Y., F.X. Luo, L. Liu and F.C. Guo (2010) *In vitro* induction of tetraploids in crape myrtle (*Lagerstroemia indica* L.). Plant Cell Tissue Organ Cult. 101: 41–47.