Plant Cell, Tissue and Organ Culture **70:** 289–293, 2002.

In vitro production and identification of autotetraploids of *Scutellaria baicalensis*

S. L. Gao, B. J. Chen & D. N. Zhu
Department of Genetics and Breeding, China Pharmaceutical University, Ji Xiang An, Nanjing, P.R. China 210038

Received 6 February 2001; accepted in revised form 2 January 2002

Key words: colchicine, medicinal plants, tetraploid

Abstract

The roots of *Scutellaria baicalensis* are a major traditional Chinese medicine. We report research on induction, characteristics and chemical analysis of polyploid plants of *S. baicalensis*. Immersing calluses in 0.2% colchicine solution for 12 h prior to culture induced a high number of tetraploid plants. The induction rate reached as high as 40% of treated calluses. More than 50 lines of tetraploid plants were obtained. All tetraploid plants showed typical polyploidy characteristics. Twenty selected tetraploid lines were transferred to the field for determination of morphological characteristics and for chemical assays. Seven elite lines have been selected for further selection and breeding into new varieties for commercial production.

Abbreviations: BA – benzylaminopurine; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; PP_{333} – paclobutrazol; B_5 – Gamborg et al. (1968) medium; MS – Murashige and Skoog (1962) medium

Introduction

The importance of polyploid plants in agriculture is well documented (Lewis, 1980). Nevertheless, only few cases of polyploid medicinal plant have been reported (Gao, 1996). Tetraploid plants of *Datura stramonium* have a 1–2 times higher alkaloid content in leaves, stems and roots as compared with diploid plants (Rowson, 1949). The content of alkaloid in tetraploid plants of *Atropa belladonna* is 154% of that in the diploid plant (Jackson, 1953). In addition, leaves, stems and roots of polyploid plants are usually bigger than those of diploid plants. Fertility and seed yield in most medicinal plants are not as important as in crop plants, because in most cases only leaves, stems and roots are used. Inducing polyploid plants by tissue culture is advantageous because of high effectivity and convenience in comparison with traditional methods (Gao et al., 1996).

The root of *Scutellaria baicalensis* is a major traditional Chinese medicine used to relieve cough, arrest bleeding and prevent abortion. Callus culture and flavonoid production *in vitro* have been reported (Yamamoto et al., 1986; Morimoto et al., 1995). To develop superior varieties of *S. baicalensis*, we previously reported preliminary results on induction of autotetraploids by tissue culture (Chen et al., 2000). In this paper, we describe further research including induction, identification of agronomic characteristics and chemical composition of tetraploid plants.

Material and methods

Plant material

Scutellaria baicalensis was identified by Professor B.Y. Yu, China Pharmaceutical University.

Callus induction

Seeds of *S. baicalensis* Georgi were sterilized in 2% sodium hypochlorite (NaOCl) for 15 min. The seeds were rinsed several times in sterile distilled water and then transferred to a Petri dish containing filter paper to remove water. The sterilized seeds were placed on 1/2 strength MS solid culture media to germinate in

the light. After 15 days, seedlings were cut into cylindrical pieces, approximately 5 mm in length. The explants were transferred to basal MS media supplemented with 0.5 mg l^{-1} BA and 0.5 mg l^{-1} IAA in the dark to induce callus.

Induction of polyploid plantlets

Thirty pieces of callus were inoculated in MS solid medium (with 0.5 mg l^{-1} BA and 0.5 mg l^{-1} PP_{333}) containing four concentrations of colchicine and cultured for 30 days in an illuminated incubator at 25°C or submerged in 0.2% (w/v) colchicine solution for short periods of time up to 24 h. Then, treated calluses were transferred to MS media supplemented with 0.1 mg l^{-1} BA and 0.5 mg l^{-1} PP_{333} and cultured in an illuminated incubator at 25°C to induce buds and plantlets. Finally, the plantlets were transferred to rooting media (solid 1/2 MS media supplemented with 0.1 mg l^{-1} NAA) to induce roots for further chromosome determination.

Chromosome determination

About 0.5 cm of the root-tips of plantlets were removed and pretreated in 0.1% colchicine solution for 30 min. The root-tips were fixed in Carnoy's fluid for 2 h, rinsed with 70% alcohol and then macerated for 5 min with 1 M HCl. The fixed root-tips were stained with Carbol fuchsin. A photomicroscope (Olympus BH-2, Japan) was used for chromosome determination. The chromosome count of each tetraploid ($4x$=36) plantlet was repeated at least three times.

Observation of agronomic characteristics

Twenty tetraploid plantlets of *S. baicalensis* were transplanted to the experimental field on the campus and eight tetraploid plantlets were selected for further identification of agronomic characteristics.

Baicalin content of tetraploid plants

Roots of all obtained tetraploid plants were harvested after growing for 8 months in the field and used to determine the contents of baicalin. Dried mixture samples (70 mg) were extracted 3 h with 40 ml solvent (MeOH) in flasks. MeOH was added to 50 ml. One ml solution was diluted with 10 ml MeOH solvent for HPLC analysis.

HPLC conditions were: C_{18}column (4.6 mm×250 mm), the elution solvent was MeOH:H_2O:H_3PO_4 (55:45:0.2), the flow rate was 1.0 ml min^{-1} and the detection wavelength was 275 nm.

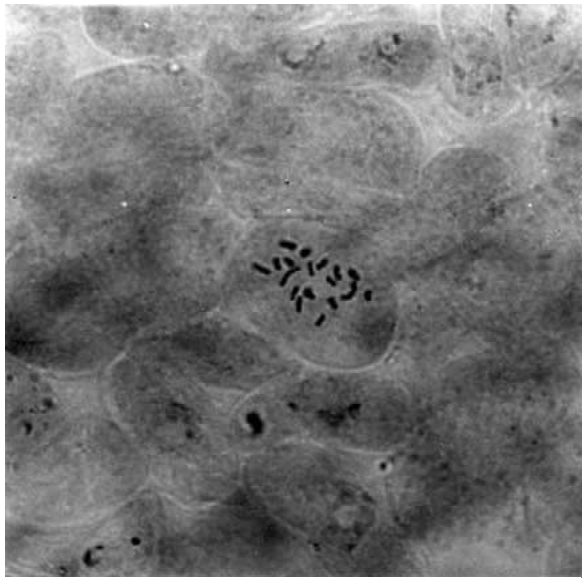

Figure 1. The chromosome of diploid plant; $2n$=$2x$=18 (×1600).

Results and discussion

Effect of colchicine concentrations on inducing tetraploidy

After treatment with increasing colchicine concentration for 30 days, the percentages of calluses that died increased with the colchicine concentration. The surviving calluses were subcultured and developed buds and/or plantlets. According to the chromosome counts, some plantlets were tetraploid (Table 1). The data in Table 1 indicate that 10 mg l^{-1} colchicine was suitable for the induction of polyploidy. The induction rate of polyploidy was 16.6%. On the other hand, immersing calluses in 0.2% colchicine solution for short time was much more efficient in inducing tetraploidy. (Table 2).

After immersion of callus in 0.2% colchicine for 12 h, 40% of the initial calluses yielded tetraploid plants. This is the highest inducing ratio in our experiments thus far. Chromosome counts revealed that the polyploid plantlets had 36 chromosome ($4x$=36) (Figures 1 and 2).

Agronomic characteristics of tetraploid plants

The experiments in the field showed that most of the tetraploid plants grew vigorously. The leaves were

Table 1. Influence of colchicine on induction of polyploidy

Colchicine (mg l^{-1})	Number of surviving calluses	Tetraploids (number)	Tetraploids (% of surviving calluses)	Tetraploids (% of initial calluses)
0	30	0	0	0
5	24	1	4.2	3.3
10	20	5	25.0	16.7
50	10	3	30.0	10.0
100	5	2	40.0	6.7

*Calluses were cultured for 30 days with colchicine. The number of inoculated callus in each treatment was 30

Table 2. The effects of immersing callus in colchicine solution on inducing tetraploid

Number of immersed calluses	Duration of immersing (h)	Number of survived calluses	Obtained number of tetraploids	Tetraploids rate (% of initial calluses)
30	0	30	0	0
30	3	30	4	13
30	5	28	6	21
30	8	23	7	30
30	12	20	12	60
30	16	13	10	77
30	24	9	6	67

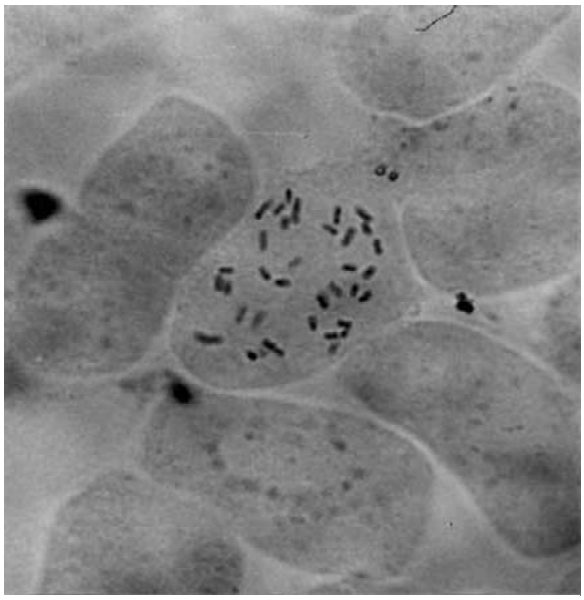

Figure 2. The chromosome of tetraploid plant; $2n=4x=36$ ($\times 1600$).

thicker, rougher and larger than those of diploid plants (Table 3). The stomata on the surface of the leaves were large but there were fewer compared to the controls. The stems and roots of polyploid plants were also larger and longer. These characteristics are typical characteristics of polyploid plants (Pei, 1985). This result means the plant weight or yield of tetraploid plants will increase greatly compared with that of the diploid plants.

Determination of major chemical compounds

The content of effective compounds is very important for medicinal plants. In order to evaluate the medicinal value of polyploid plants of *S. baicalensis*, root samples of each tetraploid plant were extracted and analyzed by HPLC. Baicalin was used as reference. Figure 3 shows HPLC profiles of the standard, control and tetraploid line D20, respectively. The retention time of baicalin was about 11.0 (Figure 3).

The contents of baicalin in each polyploid strain are shown in Table 4. The results show that only one line, D-20, showed higher content of baicalin than the control. Most of tetraploid plants had a little smaller baicalin content (%) than that in control, but most tetraploid plant showed higher productivity of baicalin (yield of baicalin per plant) than the control, due to their higher root weight.

Table 3. The identification of characteristics for tetraploids and control

Lines	Plant height (cm)	Stem diameter (mm)	Leaf width (mm)	Leaf length (mm)	Width of stomata (μm)	Length of stomata (μm)	Density of stomata
Original plant	21.2	12.1	11.0±0.4	42.1 ±1.0	7.7±1.4	15.1±1.7	54.4±2.1
B1	22.1	14.7	16.5±1.0***	48.2±2.4***	9.9±1.4***	18.1±1.9***	34.3±2.0***
B17	24.9	14.7	14.9±0.8***	44.4±1.7***	9.35±1.4**	18.0±2.3***	33.4±1.8***
C1	23.3	15.1	14.1±0.6***	46.2±1.5***	9.4±1.4***	17.1±1.8***	33.6±1.5***
C7	24.5	15.3	16.0±0.6***	47.9±2.1***	9.8±1.4***	17.9±1.9***	32.2±1.7***
D13	20.6	13.4	13.1±0.7***	42.3±1.2	8.9±1.7*	16.4±2.1*	37.5±1.8***
E1	22.2	14.5	14.4±0.7***	46.7±1.5***	9.3±1.5***	17.9±2.3***	32.2±1.9***
E12	25.2	15.2	15.8±0.9***	48.4±1.7***	9.7±1.4***	17.6±1.8***	32.4±1.6***
F8	25.8	14.8	15.8±0.9***	48.6±1.8***	10.4±1.5***	19.3±2.5***	31.4±1.7***

Original plant was diploid plant. Data are the average of 20 random samples X±SE Densities of stoma are the average of five visual fields (×400) under microscope X±SE, (*t-Test 0.05; **t-Test 0.01; ***t-Test 0.001).

Table 4. The content of baicalin in diploid and tetraploid roots of *Scutellaria baicalensis*

Lines	Dry weight (g plant^{-1}) %	Content of baicalin (%)	Yield of baicalin (g plant^{-1})	%
Original plant	1.170	16.51	0.1932	100
B1	1.485	15.68	0.2328	120.5
C1	1.438	14.69	0.2112	109.3
C7	1.898	15.02	0.2851	147.6
E1	1.515	14.34	0.2172	112.4
E12	1.786	15.37	0.2745	142.1
F8	1.525	14.18	0.2162	111.9
B17	1.757	13.98	0.2456	127.1
D13	1.266	13.17	0.1667	86.3
F5	1.360	14.23	0.1936	100.2
E16	1.431	15.98	0.2287	118.4
A1	1.234	14.24	0.1757	90.9
B3	1.255	13.86	0.1739	90.0
C2	1.713	15.27	0.2616	135.4
D4	1.413	15.18	0.2145	111.0
E10	1.563	14.81	0.2275	117.8
F4	1.491	15.52	0.2314	119.8
B16	1.821	14.90	0.2713	140.4
D5	1.575	14.39	0.2266	117.3
D20	1.555	17.27	0.2685	139.0
C8	1.283	14.10	0.1809	93.6

Original plant was diploid plant. The contents of Baicalin were based on weight of dry root.

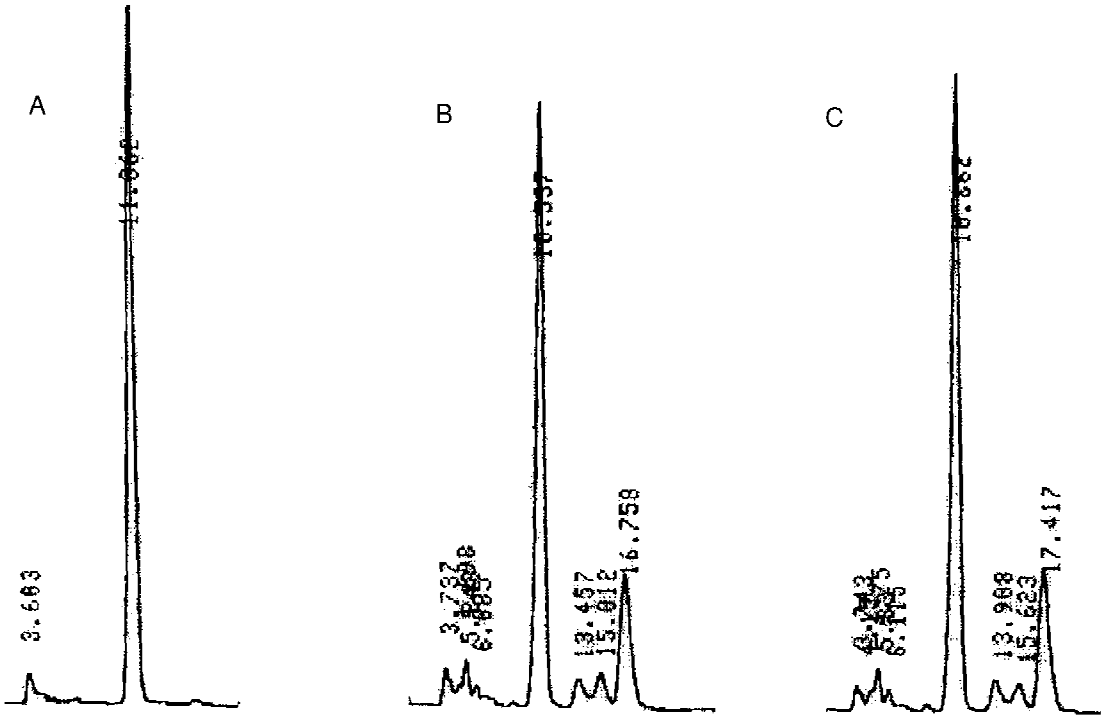

Figure 3. (*A*) HPLC trace of baicalin standards (retention time, 11.06 min); (*B*) HPLC trace of baicalin in original plant; (*C*) HPLC trace of baicalin in tetraploid line D20.

From the 20 tetraploid lines, the yield of baicalin in lines C1, E1, F8, D13, F5, E6, A1, B3, D4, E10, F4, D5, and C8 was lower or only a littler higher (<120%) than that in the control (CK). These lines were eliminated. The yield of baicalin in lines B1, B17, D20, C7, E12, C2 and B16 was higher (>120%) than that in the control, because these lines had a higher yield of roots and/or have a higher content of baicalin. These tetraploid plants will be screened for a longer period of time and used in breeding to obtain superior new varieties for the commercial production.

Acknowledgments

This research had been financed by a grant from National Administration Department of Traditional Chinese Medicine, P.R. China.

References

Chen BJ, Gao SL & Bian YY (2000) The induction of autotetraploids of *Scutellaria baicalensis* Georgi by tissue culture. J. Plant Res. Environ. 9: 9–11

Gao SL, Zhu DN, Cai ZH & Xu DR (1996) Autotetraploid plants from colchicine-treated bud culture of *Salvia miltiorrhiza* Bge. Plant Cell Tiss. Org. Cult. 47: 73–77

Jackson BP & Rowson JM (1953) Alkaloid biogenesis in tetraploid *Stramonium*. J. Pharm. Pharmacol. 5: 778–793

Lewis WH (1980) Polyloidy: Biological Relevance. (pp 180–182) Plenum Press, New York

Morimoto S, Harioka T & Shoyama Y (1995) Purification and characterization of flavone-specific beta-glucuronidase from callus culture of *Scutellaria baicalensis* Georgi. Planta 195: 535–540

Pei XS (1985) Polyploidy Induction and Breeding. (pp 95–98) Shanghai Science and Technology Press

Rowson JM (1949) Increased alkaloid contents of induced polyploid of *Datura.* Nature 154: 81–82

Yamamoto H, Chatani N & Kitayama A (1986) Flavonoid production in *Scutellaria baicalensis* callus cultures. Plant Cell Tiss. Org. Cult. 5: 219–222