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Autotetraploid plants from colchicine-treated bud culture of *Salvia miltiorrhiza Bge*

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Abstract

The root of *Salvia miltiorrhiza* is a traditional Chinese medicinal plant used as an important drug to cure cardiovascular diseases. Reseacch on the technology of induction, identification and chemical analysis of polypoid plants is reported. The obtained results indicated that basal MS media plus 10 ppm colchicine can induce polyploid mutants effectively. Tetraploid plants were transferred to the fields so that comparative experiment for further identification, observation and screening of 15 agronomic characteristics could be made. The major chemical compounds, tanshinones, in two tetraploid plants were higher than that in the control. An excellent plant 61-2-22 may develop into a new variety for large scale production.

Abbreviations: BA – bezylaminopurine; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; B_5 – Gamborg et al. (1968) medium; HPLC – high pressure liquid chromatography; MS – Murashige and Skoog (1962) medium

Introduction

The significance and importance of the polyploid plants in agriculture had already been adequately demonstrated (Lewis, 1980). Despite considerable research on polyploid plants, only a few cases of polyploid medicinal plant have been reported. It has been reported that the tetraploid plant of *Datura stramonium* contained 1-2 times higher alkaloid content in the leaf, stem and root compared with that in the diploid plant (Rowson, 1949). The content of alkaloid in the tetraploid plant of *Atropa belladonna* was 153.6% of that in the diploid plant (Jackson and Rowson, 1953).

In addition, the leaf, stem and root. which can be useful parts in most medicinal plant, in polyploid plant are usually bigger than those of the diploid plant. Thus the polyploid plants may increase the biomass or product yields.

The root of *Salvia miltiorrihiza* is a major traditional Chinese medicine used for cardio-vascular diseases. In this paper we describe the induction, identification and the chemical assay of tetraploid plants of *Salvia miltiorrihiza*.

Material and methods

Plant material

Salvia miltiorrihiza was supplied by The Medicinal Botany Garden of China Pharmaceutical University.

Culture procedure

The seeds of *Salvia miltiorrihiza* 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 Waterman No.50 filter paper to remove water. The sterilized seeds were placed on 1/2 strength basal MS solid culture media to germinate under light. After 15 days the seedlings were transferred to basal MS media supplemented with BA 1 mg l^{-1} and IAA 0.5 mg l^{-1} to induce bud clumps in an illuminated light intensity 36μmol $m^{-2}s^{-1}$ incubator at 25 °C.

Table 1. Influence of colchicine in inducing polyploidy.

No. of treatments	Concentration of colchicine	Growth habit	Tetraploids obtained
61-0	0 ppm	normal	0
61-1	5 ppm	inhibited	2
61-2	10 ppm	30% buds died	12
61-3	50 ppm	60% buds died	6
61-4	100 ppm	80% buds died	2

The number of inoculated buds in each treatment was 100

Table 2. The agronomic characteristics of control and tetraploid plants of *Salvia miltiorrhiza*.

Lines	Plant type	Plant height	Growth potential	Stem diameter	Leaf thickness	Density of stoma	Size of stoma
Control	loose	43.27±1.11	Normal	0.49±0.01	Normal	19.55±0.53	10.03×2.03
61-2-22	middle	51.35±1.28	Strong	0.48±0.02	Thicker	16.75±0.49	12.63×2.28
61-3-6	middle	51.76±1.74	Normal	0.52±0.02	Thicker	13.28±0.36	12.31×2.16
61-4-3	compact	35.60±0.47	Normal	0.53±0.03	Thicker	11.95±0.34	13.45×2.65
62-5	compact	59.9±1.77	Strong	0.46±0.02	Thickest	13.50±0.45	13.75±2.88
62-6	compact	59.69±0.96	Strong	0.52±0.01	Thicker	12.40±0.35	12.70×2.93
62-50	compact	59.89±0.81	Strong	0.51±0.01	Thicker	11.35±0.36	13.63×2.35
62-91	compact	50.60±1.72	Normal	0.43±0.02	Thicker	10.65±0.33	13.00×2.20
62-98	compact	51.41±1.07	Strong	0.52±0.03	Thicker	11.90±0.49	12.98×2.40
62-129	compact	48.68±4.07	Normal	0.46±0.04	Thicker	12.20±0.35	13.00×2.38
62-166	compact	55.73±0.63	Normal	0.45±0.02	Thicker	12.85±0.48	13.68×2.50

Lines	Date	Fertility	Floral length	Root color	Root branch	Root diameter	Root length	Fresh weight
Control	2/5	Normal	22.25±0.45	red-yellow	4-10	1.31±0.09	20.7±1.71	98.15±6.01
61-2-22	4/5	semi-sterile	22.51±0.69	purple-red	10-14	0.99±0.06	27.45±0.91	161.60±10.68
61-3-6	8/5	semi-sterile	21.36±1.07	Red	5-10	1.45±0.05	27.40±1.69	169.00±4.02
61-4-3	8/5	semi-sterile	8.05±0.13	Red	5-10	1.83±0.05	24.85±1.45	189.80±10.23
62-5	1/6	semi-sterile	16.42±0.68	purple-red	8-12	1.44±0.04	38.40±1.59	257.75±15.53
62-6	15/6	semi-sterile	18.55±0.77	purple-red	8-10	1.68±0.07	38.35±1.73	230.95±12.48
62-50	15/6	semi-sterile	19.87±0.90	purple-red	8-12	1.72±0.07	34.70±1.78	273.65±20.19
62-91	30/5	semi-sterile	10.75±1.11	Red	5-10	1.69±0.13	34.08±1.31	136.70±9.59
62-98	20/5	semi-sterile	11.99±0.95	Red	8-10	1.76±0.07	36.47±0.88	223.58±13.06
62-129	30/6	semi-sterile	15.48±2.36	Red	5-8	1.81±0.59	31.30±1.52	150.95±15.03
62-166	30/6	semi-sterile	15.58±1.40	purple-Red	8-10	1.69±0.04	36.85±1.55	188.85±12.14

The data of floral height (cm), root diameter (cm), root length (cm) and fresh weight (g) of per root was average of 20 random plot samples X±SE

Induction of polyploid plantlets

The bud clumps were inoculated in MS medium containing four concentrations of colchicine (5, 10, 50, 100 ppm) in order to induce polyploid variations. After 30 days, the surviving buds and plantlets were transferred to MS media supplemented with BA 1 mg l^{-1} and IAA 0.5 mg l^{-1} in an illuminated incubator at 25 °C. Finally, the plantlets were transferred to rooting media (B_5 solid media containing IBA 0.2 mg l^{-1}) to induce roots for further chromosome determination.

Chromosome determination

About 0.5 cm of the root-tips of plantlets were removed and pretreated 0.1% colchicine solution for one and half hours. The root-tips were fixed in Carnoy's fluid for 2 h, treated with 70% alcohol for 80 min and then

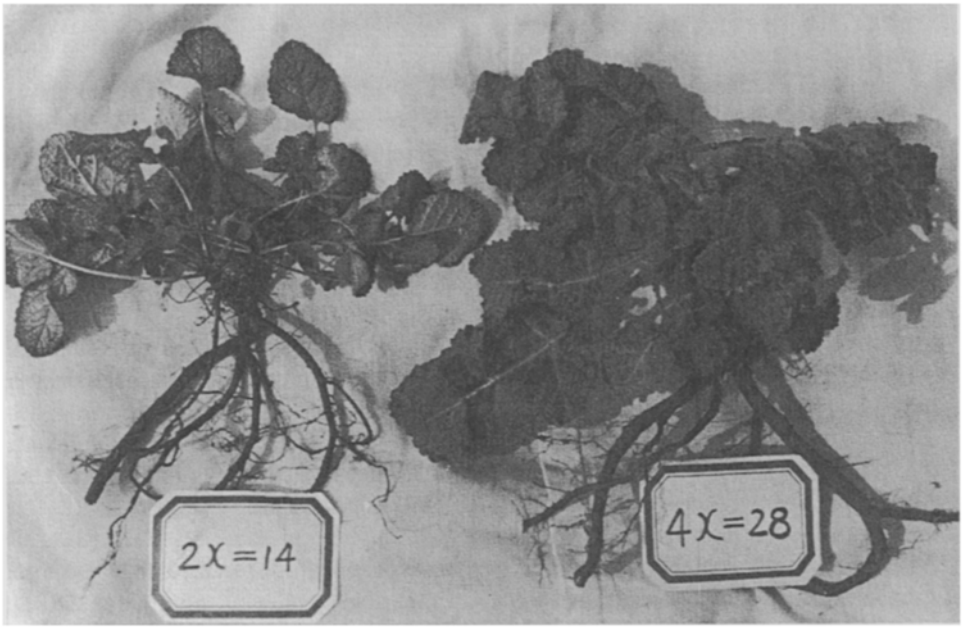

Figure 1. Diploid plant (left) and Tetraploid plant (right) of *Salvia miltiorrhiza*.

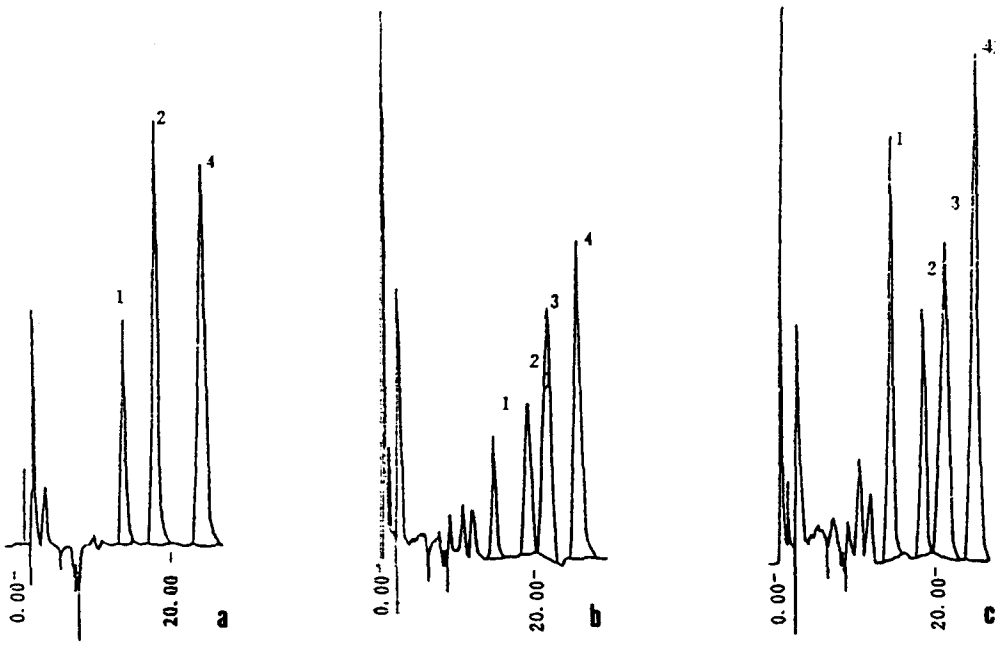

Figure 2. (*a*) HPLC trace of three standards; (*b*) HPLC trace of control; (*c*) HPLC trace of 61-2-22. 1: Cryptotanshinone (retention time, 15.2 min); 2: Tanshinone 1 (retention time, 19.2 min); 3: Unknown compound; 4: Tanshinone HA (retention time 25.4 min).

macerated with mixed acid (45% acetic acid : 1% HCl : 1% H_2SO_4; 100:10:1). The fixed root-tips were stained with Carbol fuchsin. The photomicroscope (Olympus BH-2) was used for chromosome determination. The chromosome count of each tetraploid (4x=28) plantlet was repeated at least three times.

Observation of agronomic characteristics

All tetraploid plantlets of *Salvia miltiorrihiza* were transplanted to the experimental plots for further identifcation of agronomic characteristics for two years. Ten superior polyploid plants were selected for further comparison using 15 agronomic characteristics.

Chemical assay of tetraploid plant

Roots of tetraploid plants were harvested in November and used to determine the contents of tanshione I, IIA and cryptodashinone respectively. Ground dry samples (0.5 g each) were extracted twice with a 10 ml solvent mixture (CH_2Cl_2 : MeOH; 8:2) in 50 ml Erlenmeyer flasks on a shaker (150 rpm) overnight at room temperature. The combined extracts were evaporated to dryness and redissolved in 10 ml solvent for HPLC analysis. HPLC conditions were; a C_{18} column (5 mm × 250 mm), the elution solvent was MeOH : H_2O (75:25), the flow rate was 1.0 mg min^{-1} and the detection wavelength was 254 nm.

Results

Effect of colchicine concentrations on the inductions of tetraploidy

One hundred buds were inoculated onto each medium containing different colchicine concentrations and cultured for 30 days. About half of the buds died, but the surviving buds were subcultured and developed into plantlets. According to the chromosome determination, some of the plantlets were tetraploidy.

Table 1 indicates that 10-50 ppm colchicine in media was suitable for the induction of polyploidy. The induction rate of polyploidy was as high as 6-12%. The chromosome number of polyploid plantlets was 28 (2n=4x=28)

Variation of agronomic characteristics in tetraploid plants

According to the results of comparative experiments in the field plots, most of the tetraploid plants grew vigorously. The leaves were thicker, rougher and larger than those of diploid plants (Table 2). The stomata on the surface of the leaves were also bigger and fewer in number than those of the controls. The stems and roots of polyploid plants were also larger and longer with dark color (Figure 1).

On the other hand, all the tetraploid plants were semisterile and showed typical characteristics of polyploid plant (Pei, 1985). The average fresh weight of the root in tetraploid plants increased greatly (1.54-2.79 times) compared with that of the original plant of *Salvia miltiorrhiza.*

The high productivity of the root, which was the most useful part for medicinal purpose, could have important significant and economical value.

Determination of major chemical compounds

The content of effective compounds is very important in the medicinal plant. A root sample of each tetraploid plant was extracted and analyzed by HPLC. Three standard compounds were used as a reference. Figure 2a,b,c show the HPLC profiles of the three standards, control and tetraploid strain 61-2-22 respectively. The retention time of cryptotanshinone, tanshinone I and tanshinone IIA were 15.2, 19.2 and 25.4 min, respectively.

The contents of these compounds in each polyploid strain are presented in Table 3. The result show that only two plants 61-2-22 and 61-3-6 showed higher content of tanshinones than the control. Six tetraploid lines (61-2-22, 61-3-6, 62-5, 62-6, 61-4-3, and 62-166) showed much high productivity of tanshinone (total yield of tanshinones / plot) than the control, because of their high root yields (per plot, 20 m^2).

Off the ten selected tetraploid plants, 61-2-22 was found to be the best plant, not only in the high yield of root but also in the high content of major compounds.

Discussion

Polyploid plants usually have larger and thicker leaves, stems or roots, which will increase yields of the medicinal plants. Fertility and seed yield in most medicinal plant are not as important as that in crop plants, because

Table 3. The content of tanshinones and cryptotanshinone in control and tetraploid roots of *Salvia miltiorrhiza.*

Lines	Tanshinone I (%)	Tanshinone II A(%)	Cryptotanshinone (%)	Total (%)	Dry/fresh root (%)	Average root yield/plot(kg)	Total weight (g) of tanshinones/plot
Control	0.0959	0.1682	0.1593	0.4234	26.62	6.53	27.65
61-2-22	0.1266	0.2063	0.4239	0.7568	27.64	11.17	84.53
61-3-6	0.0965	0.1174	0.2472	0.4611	29.73	12.56	57.91
61-4-3	0.0542	0.0836	0.1167	0.2545	28.19	13.38	34.05
62-5	0.0654	0.0981	0.1846	0.3481	23.28	15.00	52.22
62-6	0.0568	0.0923	0.1176	0.2667	28.68	16.56	44.17
62-50	0.0415	0.0618	0.0820	0.1854	20.94	13.95	25.86
62-91	0.0410	0.0777	0.0907	0.2094	34.16	11.67	24.44
62-98	0.0388	0.0536	0.0940	0.1872	24.24	13.55	25.37
62-129	0.0392	0.0531	0.0839	0.1762	26.32	9.93	17.50
62-166	0.0543	0.0726	0.1261	0.2530	25.16	11.88	30.06

The contents of tanshinone I, IIA and cryptotanshinone were based on weight of dry root. Plot area 20 m^2

in most cases, leaf stem and root are used for medicinal purposes.

The advantage of inducing polyploid plants by tissue culture will be as follows:

- A large number of bud, seed or seedling can be treated effectively and accurately in tissue culture by adding colchicine or other mutagens to prepared media.
- The determination of chromosome number in the roots of polyploid plants *in vitro* is very convenient and effective in comparison with those of field plants.
- Selected polyploid plant material may be used for rapid-propagation by tissue culture for further field identification and commercial production.

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