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Full Length Research Paper

Cell line screening of *Catharanthus roseus* for high yield production of ajmalicine

Bei-Bei Xiang, Ye-Rong Zhu, Wen-Juan Wang, Yan-Ling Bai and Yong Wang*

College of Life Science, Nankai University, 300071 Tianjin, P. R. China.

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Cell line screening of *Catharanthus roseus* for high yield production of ajmalicine was conducted through subculturing of callus that induced from tetraploid plant leaves. The cell line was characterized as green colour with photosynthetic capacity. The content of ajmalicine reached 2.578 and 0.895 mg/g DW in callus and in cell suspension culture, respectively. The cell suspension culture treated with methyl jasmonate (MeJA) harvested a drastic increase of ajmalicine yield (2.2 mg/g DW in cell and 2 mg/L in culture medium). Therefore, the cell line had potential commercial value.

Key words: *Catharanthus roseus*, ajmalicine production, callus culture, cell suspension culture, methyl jasmonate.

INTRODUCTION

Ajmalicine, first isolated from yohimbe bark, is one of the principal alkaloids of *Catharanthus roseus* and widely used to treat circulatory disorders. Approximately 3600 kg are produced each year at a market price of about $2000/kg (David et al., 1995). *C. roseus* cell culture has been investigated and is considered to be a potential means to produce ajmalicine, as it can accumulate more ajmalicine than in the intact plants (Hirata et al., 1987). To date, several high ajmalicine production cell lines or callus cultures have been reported (Zhao and Verpoorte, 2007). These cell lines were mainly obtained by selection of subcultures of calli. Mutation of *C. roseus* plants has been investigated to improve the productivity of target secondary metabolites (Moreno et al., 1995), but little attention has been paid to the production of ajmalicine.

It has been confirmed that polyploids of medicinal plants are usually valuable because of more biomass or higher content of effective compounds (Huang et al., 2008). Tetraploid *C. roseus* plants have been show that the content of alkaloid varies largely among individuals, which may be attributed to their genetic investigated for production of alkaloids and the results differences (Kulkarni et al., 1984; Goswami et al., 1996).

In this study, we induced callus from a tetraploid leaf of *C. roseus*. Measurement of the alkaloids in the callus showed a very high accumulation of ajmalicine. Therefore, cell suspension culture was introduced and treated with methyl jasmonate, in order to further increase the production of ajmalicine.

MATERIALS AND METHODS

Production of tetraploid plants

The *C. roseus* seeds were directly soaked in 0.04% (v/v) aqueous solution of colchicine (sigma, USA) for 48 h at room temperature and sown in pots in glasshouse after rinsing with sterile distilled water for five times. The seedlings were transplanted into field after one-month growth, among which only those plants with morphological differences in leaves were selected for further chromosome analysis by means described preciously (Chen et al., 1986), to identify tetraploid individuals (4n = 32).

Maintenance of callus, and cell suspension culture

C. roseus callus was established from leaf explants of both the tetraploid plant and the diploid control. Callus was induced in Murashige and Skoog (MS) medium supplemented with 0.5 mg/L α-naphthaleneacetic acid (NAA), 0.5 mg/L 2, 4-dichlorophenoxyacetic acid, 2 mg/L 6-benzylaminopurine, 30 g/L sucrose and 7.2 g/L agar

*Corresponding author. E-mail: wangyong@nankai.edu.cn.

in 50 ml flasks in the illumination with light intensity of 2500-3000 Lux and light period of 16 h/day at 23 ± 2°C. After three-week inducation, calli growing along the incisions were excised and transferred to solid MS medium containing 2 mg/L NAA and 1 mg/L kinetin (KT), 30 g/L sucrose and 7.2 g/L agar. Stable T1 and D1 callus lines were obtained from the tetraploid and the diploid leaf, respectively, after several rounds of subcultures with each interval of 30 days.

For cell suspension culture, cultures were maintained in 50 ml liquid MS medium containing 2 mg/L NAA, 1 mg/L KT and 30 g/L sucrose in 250 ml Erlenmeyer flask. The cell suspension culture subculturing was performed by adding fresh medium (100 ml) to 14-day-old suspension cultures (50 ml) in 250 ml Erlenmeyer flask. The cell-medium mixture was then evenly distributed into three 250 ml Erlenmeyer flasks, each containing 50 ml of suspensions. The cultures were shaken at 80 rpm and maintained in the illumination with light intensity of 2500 to 3000 Lux and light period of 16 h/day at 23 ± 2°C.

Chlorophyll content analyses

Chlorophyll content was determined after 30 days growth. Calli (2 g) was extracted with 10 ml 95% ethanol at 25°C overnight. Chlorophyll in the supernatant was quantified with a microplate spectrophotometer (UV754N, Shanghai, China) at 665, 649 nm. Chlorophyll a: Ca = 13.95 A665 - 6.88 A649; chlorophyll b: Cb = 24.96 A649 - 7.32 A665; Ca+b = Ca + Cb. Assays were carried out three times on duplicate samples. Blank control was 95% (v/v) ethanol. Dry weights were also determined.

Measurements of oxygen evolution

Oxygen evolution was measured with a Clark-type oxygen electrode (Chlorolab-2, Hansatech). Temperature was controlled at 25°C with a Polystat Refrigerated Bath (Cole-Parmer Instrument Co., Vernon Hills, IL, USA). Illumination was provided by a light housing and stabilized power supply (LS2; Hansatech Instruments Ltd.) and light was passed through a fibre-optic cable (A8; Hansatech Instruments Ltd) to the surface of reaction cuvette. Irradiance was measured with a quantum sensor (QRT1; Hansatech Instruments Ltd.) and controlled by neutral density filters. Net photosynthesis rate was determined by measuring oxygen production using a Clark-type oxygen electrode (Chlorolab-2, Hansatech), and thermostated at 25°C by a temperature controller with a Polystat Refrigerated Bath (Cole-Parmer Instrument Co., Vernon Hills, IL, USA) connected to the water jacket of the electrode. Calli of 0.5 g fresh weight were resuspended in the MS liquid medium, and added with 1 mmol/L $NaHCO_3$ as the inorganic carbon source. Photosynthetic O_2 evolution was started by illumination. The photon irradiance was 1200 µmol E/m^2/s. Light measurement was carried out with a LI-188B integrating radiometer using a spherical quantum sensor LI-193SA (LI-COR, Nebraska, USA). The O_2 electrode system was calibrated daily with sodium dithionite-treated MS liquid medium. The respiratory rate was estimated as O_2 consumption in darkness.

Alkaloid extraction and HPLC analysis

Alkaloid extraction and determination was carried out as described previously. HPLC conditions: Shimadzu LC-4A instrument with SPD-2AS UV-detector, Kromasil ODS C18 column (250 × 4.6 mm, 7 µm); samples were eluted with a mobile phase of methanol-0.005 mol/L monoammonium phosphate (70:30, v/v, pH = 7.2) at a flow rate of 1.5 ml/min. The detection wavelength was 254 nm. Retention time of five alkaloids: vindoline (9.8 min), catharanthine (13.1 min), ajmalicine (15.2 min), unknown alkaliod 19T (19 min), unknown alkaliod 31T (31 min).

Alkaloid identification by LC/MS

For mass spectrometric identification the samples were dissolved in freshly prepared methanol and analyzed by an Agilent Technologies 6520 Accurate-Mass Q-TOF LC/MS equipped with ESI ion source. The elution solvent for LC/MS was acetonitrile-0.1 mol/L ammonium acetate (49:51, v/v) and the flow rate was 0.5 ml/min. Electron impact ionization at 175V voltage was used for the MS.

Statistical analyses

Results of the physiological parameters were analyzed by using analysis of variance (ANOVA) (SPSS 13.0). Significant differences from control values were determined at $P<0.05$ level. All the results were represented as mean ± SD of at least three independent replications.

RESULTS AND DISCUSSION

Different characteristics in D1 and T1 calli

The D1 callus had a pale yellow colour and fragile quality, whereas the T1 callus was green and more compact (Figure 1). As shown in Table 1, the growth of the latter was slower than the former. Interestingly, the T1 culture turned green only after 5 rounds of subcultures and remained green thereafter. Measurement of photosynthetic evolution of oxygen showed that the callus had high photoautotrophic capacity (Table 1), which should be valuable in conservation of energy in the industrial application.

Alkaloid accumulation in calli

As shown in Table 2, the leaves could stably synthesize higher amount of catharanthine and vindoline, but lower amount of ajmalicine. In contrary, callus accumulated much more ajmalicine than catharanthine and vindoline, which was in agreement with early studies (Zhao et al., 2001). T1 callus synthesized 0.79 mg/g DW ajmalicine, which was about 6.5-fold higher than D1 callus, and over 26-fold higher than tetraploid leaves. Possibly, the chromosome doubling increased gene expression and enzyme activities corresponding to intermediate steps in alkaloids biosynthetic pathway (Lavania et al., 1988). However, it cannot be ruled out that the T1 culture may be a mutation during the maintenance and was selected out accidently.

Light has been reported to affect the accumulation of

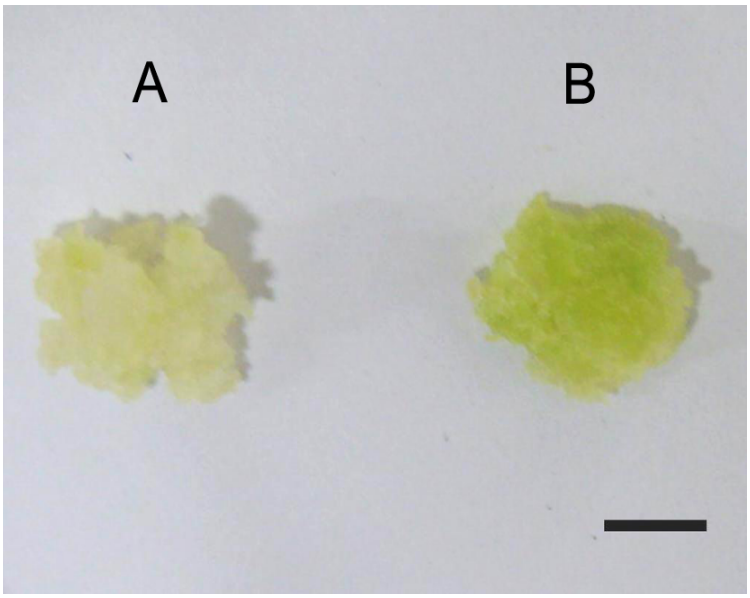

Figure 1. Photos of the D1 and T1 callus. A: D1 callus induced from the diploid leaf. B: T1 callus induced from the tetraploid leaf (bar =1 cm).

Table 1. Characteristics of the D1 and T1 callus induced from the diploid and tetraploid leave.

	D1 callus*	T1 callus*
Dry weight per flask (g)	0.31 ± 0.03a	0.38 ± 0.03b
Chlorophyll a content (μg/g DW)	0.22 ± 0.03a	0.49 ± 0.03b
Chlorophyll b content (μg/g DW)	0.06 ± 0.01a	0.17 ± 0.02b
Chlorophyll a+b content (μg/g DW)	0.28 ± 0.04a	0.66 ± 0.04b
Net photosynthesis rate (μmol O_2 /min/mg Chl)	-0.004 ± 0.0003a	0.002 ± 0.0002b
Respiratory rate (μmol O_2 /min/mg Chl)	0.012 ± 0.002a	0.005 ± 0.0005b

*The D1 callus and T1 callus were induced from the diploid and tetraploid leave respectively. Both maximum dry weight of cultures were found after 30-day culture and 15 rounds of subculture. Values were means ± SD from two independent experiments, each with three replicates. Different small letters (a, b) represented statistical significances at the P<0.05 level.

Table 2. Content of alkaloids in four different plant tissues.

Plant tissues	Alkaloid content (mg/g DW)				
	Ajmalicine	Catharanthine	Vindoline	19T**	31T**
D1 callus	0.12 ± 0.01a	0.06 ± 0.003a	nd*	1.95 ± 0.01a	0.87 ± 0.02a
T1 callus	0.79 ± 0.03b	0.21 ± 0.01b	0.03 ± 0.01a	3.85 ± 0.07b	9.64 ± 0.04b
Diploid leaves	0.05 ± 0.002c	9.82 ± 0.63c	4.39 ± 0.45b	0.51 ± 0.01c	nd*
Tetraploid leaves	0.03 ± 0.002c	13.1 ± 0.38d	4.89 ± 0.12c	0.43 ± 0.01c	nd*

Both of the calli were 30 days old, and the subculture rounds were 25. The ages of diploid and tetraploid leave were 60 days old. nd* refers to non-detectable alkaloids. **19T and 31T were the not yet fully-identified compounds, both of which showed the same molecular ion $[M+H]^+$ at m/z 353 by ESI. Their contents were determined using ajmalicine as the refered standard. Values are means ± SD from two independent experiments, each with three replicates. Different small letters (a, b, c and d) indicated statistical significances at the P<0.05 level.

ajmalicine negatively (Zhao et al., 2001). Therefore, we analyzed the content of ajmalicine in T1 callus in the darkness. The results showed (Table 3) that the ajmalicine level could reach 2.578 mg/g DW, which was higher than the high-ajmalicine-yield cell line CR6B treated by various chemicals (Zhao et al., 2000). To our knowledge, such a high production of ajmalicine has not been reported before. And therefore, this T1 callus could

Table 3. Contents of alkaloids in D1 and T1 callus in light or darkness.

Culture	Treatment	Alkaloid content (mg/g DW)				
		Ajmalicine	Catharanthine	Vindoline	19T	31T
D1 callus	Light	0.08 ± 0.01b	0.02 ± 0.001a	nd*	0.77 ± 0.04a	1.04 ± 0.07a
	Dark	0.15 ± 0.01c	0.03 ± 0.002b	nd*	0.67 ± 0.03a	0.82 ± 0.01a
T1 callus	Light	0.73 ± 0.03a	0.05 ± 0.001c	0.02 ± 0.002a	19.57 ± 0.02b	30.39 ± 0.09b
	Dark	2.58 ± 0.06d	0.04 ± 0.002c	0.01 ± 0.001a	17.61 ± 0.1c	31.86 ± 0.09c

Both of the two cultures were 30 days old, and the subculture rounds were 30. nd* refers to non-detectable alkaloids. Values are means ± SD from two independent experiments, each with three replicates. Different small letters indicate statistical significances at the P<0.05 level.

Table 4. Comparison of alkaloid contents between callus and cell suspension culture of T1 line.

Culture type	Alkaloid content (mg/g DW)				
	Ajmalicine	Catharanthine	Vindoline	19T	31T
T1 Callus	0. 73 ± 0.03a	0.19 ± 0.04a	0.01 ± 0.001a	9.18 ± 0.92a	24.69 ± 0.73a
T1 Cell suspension culture*	0.91 ± 0.07b	0.14 ± 0.02a	0.03 ± 0.003b	6.85 ± 0.41b	15.09 ± 0.47b

*T1 Cell suspension culture was initiated by transferring 1 ± 2 g of callus tissue into 50 ml liquid MS medium containing 2 mg/L NAA and 1 mg/L KT, 30 g/L sucrose in a 250 ml Erlenmeyer flask. Callus was 30 days old, and cell suspension culture was 14 days old, the subculture rounds were 30. Values are means ± SD from two independent experiments, each with three replicates. Different small letters indicate statistical significances at the P<0.05 level.

be valuable for initiation of cell cultures.

In addition, the callus cultures had two unknown products (named 19T and 31T), both of which showed the same molecular ion $[M+H]^+$ at m/z 353 by ESI. Their contents showed 2-5 folds higher in T1 culture than in D1 culture (Table 2), and were not affected by light or dark treatment (Table 3). From the literatures we know that 19-epi-ajmalicine, tetrahydroalstonine and 16-hydroxytabersonine all have a molecular weight of 352. Therefore, the 19T and 31T may be two of these alkaloids, which were considered to be the important intermediate products in the TIA biosynthetic pathway.

Effect of MeJA on alkaloid production in T1 cell suspension culture

The alkaloid production in cell suspension culture could reach the same level of the callus culture (Table 4). The results suggested that the T1 cell suspension culture may be superior to the compact callus clusters suspension culture (CCCs), which produced lower alkaloids than CCC (compact callus clusters culture) (Zhao et al., 2001). Thus, it was useful to study more about T1 cullture. As MeJA has been proved to effectively improve alkaloid production in *C. roseus* cell cultures (Rijhwani and Shanks, 1998), we also studied the effect of MeJA on the yield of ajmalicine production in T1 cell suspension culture. Our results showed that the level of ajmalicine in the cells was raised to 2 to 3 folds by MeJA treatment (Figure 2A). Moreover, catharanthine also showed a 3 to 4 folds higher level than the control (P<0.05).

Previously it has been shown that most of the alkaloids were excreted into culture medium after treatment of the cell culture with MeJA (Lee-Parsons et al., 2004). Similar results were obtained in T1 cell suspension culture (Figure 2B). The ajmalicine content in the medium was 2 mg/L, which was 10-fold higher than the control. MeJA treatment also induced the excretion of 19T and 31T, suggesting that the biosynthesis of these two alkaloids were related to the MeJA regulation.

In conclusion, we found that the T1 culture was a high-ajmalicine-yield line, which also had the higher level of vindoline, 19T and 31T. The T1 cell culture was also sensitive to MeJA treatment, with dramatic increase in the accumulation of these alkaloids both in cells and in the culture medium. Thus, we will continue to improve the alkaloids yield by treating with other various abiotic and biotic elicitors or signal molecules. Due to the photoautotrophic capacity of T1 culture, we expect to study the growth conditions in bioreactor with great interest. These results may contribute to more profound understanding on indole alkaloid biosynthesis and its regulation, and also lead to the enhancement of indole alkaloid production in cell cultures.

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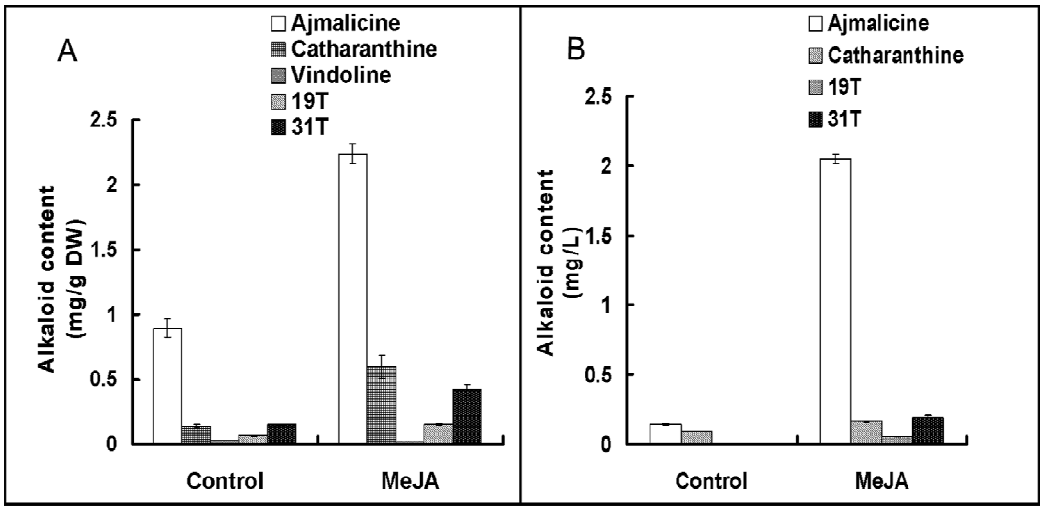

Figure 2. Effects of MeJA on alkaloid accumulation in *C. roseus* T1 cell suspension culture and culture medium. A: Alkaloid content in cells. B: Alkaloid content in the culture medium. 100 μM MeJA was added to 8-day-old cell suspension culture and cultivated in the illumination with light intensity of 2500-3000 Lux and light period of 16 h/d at 23 ± 2°C for further 3 days. Control received the same volume of ethanol. Bars indicate standard errors (n = 3).

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