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RESEARCH ARTICLE

Establishment of cell suspension line of *Populus tomentosa* Carr.

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Abstract Leaves of fine *Populus tomentosa* genotype TC152 were used as explants to establish cell suspension lines. The effects of plant growth regulators on callus induction and establishment of cell suspension lines were studied. The callus induction rate was the highest on a MS solid medium supplemented with 1.0 mg·L^{-1} 2,4-D. A cell suspension line could be obtained by inoculating calli which were not subcultured into a MS liquid medium supplemented with 1.5 mg·L^{-1} 2,4-D. The best subculture medium was MS + 0.8 mg·L^{-1} 2,4-D + 30 g·L^{-1} sucrose with a subculture cycle of seven days.

Key words *Populus tomentosa*, callus, cell suspension line

1 Introduction

Populus tomentosa Carr., a native species in northern China, is mainly used for greening of the landscape or for fiber and wood production. With the rapid developments of cell engineering, studies using suspension cells as material are gradually being conducted. A fine cell suspension line is not only a good receptor for gene transfer (Zheng et al., 2005), but also a suitable system for studying cytology, molecular biology (Chen et al., 2002), protoplast culture, production of secondary plant metabolites (Spela et al., 2005) and somatic embryogenesis. The development of the genome database of model *Populus* species and the presence of the protocols of cell suspension cultures will provide an opportunity for studying the genes about growth and development of *Populus*. On the basis of cell suspension lines, Li and Tian (2004) analyzed the expressions of genes related to spreading of cells and cellulose synthesis. Zhao et al. (2000) investigated salt tolerance of suspended cells of *P. tomentosa*. However, there are no detailed reports about the factors affecting the establishment of cell suspension lines of *P. tomentosa* until now.

TC152 an excellent genotype of *P. tomentosa*, was used as research material to study the factors affecting the establishment of cell suspension lines and the growth characteristics of cell lines. A stable cell suspension line of *P. tomentosa* was established in our study. It is the basis for research on protoplast culture and somatic embryogenesis of *P. tomentosa*.

2 Materials and methods

2.1 Plant materials

Aseptic shoots kept *in vitro* were used as explants, called TC152. The genotype was selected from the gene bank of *P. tomentosa* in the Guanxian nursery, Shandong Province of China. TC152 has many good characteristics, such as fast growth, fine wood quality, and strong resistance to stress.

2.2 Methods

2.2.1 Callus induction

New leaves of aseptic shoots were cut into 0.5 cm × 0.5 cm as explants, which were inoculated on a callus induction media containing a solid, basal MS medium and different concentrations of BA and NAA for callus induction. Calli that were not browning were selected for subculture.

2.2.2 Suspension culture

The exuberant calli were transferred into 150 mL flasks containing 80 mL liquid media. The flasks were placed on a rotary shaker at a speed of 110 r·min^{-1}. The initial media were a MS liquid medium supplemented with different concentrations of 2,4-D.

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At the early stage of cell suspension culture, the subculture cycle was 12 d. During this subculture, cells were, at first, sretained for 2 h. The medium of the upper layer was dumped. Cells on the lower layer were filtered to remove liquid medium. And 2.0 g fresh weight cells were partitioned into 150 mL flasks containing 80 mL subculture medium.

2.2.3 Measurement of proliferation cells

Packed cell volume (PCV): Cells were resuspended fully, dumped into a 10 mL scaled centrifuge tube and centrifuged at a speed of 1000 r·min^{-1} for 10 min. The volume of compact cells in the centrifuge tube is referred to as PCV.

Fresh weight (FW): Suspension cells were, at first, meshed by a 60 mesh and then filtered. The weight of cells on the filter paper was the fresh weight of suspension cells.

2.2.4 Media and culture conditions

A MS (Murashige and Skoog, 1962) medium was used as a basal medium. The solid medium was a MS medium supplemented with 5 g·L^{-1} agar and 30 g·L^{-1} sucrose. A liquid medium was also a MS medium supplemented with 30 g·L^{-1} sucrose. The calli and cells were cultured under a 16-h light/8-h dark regime at a constant temperature of 25°C and an illumination of 500–1000 lx.

2.2.5 Statistical analysis

A two-factor, three-level experiment was designed to select the best callus induction medium. Single factor experiments were designed to study the factors that affect cell suspension culture. The experiments were replicated three times. In addition, each treatment was inoculated in five flasks. The results, expressed in percentages, were transformed by the expression $\theta=\arcsin\sqrt{\text{percentage}}$. An analysis of variance was carried out and differences between the means of the treatments were determined by LSD tests at a 0.05 level of significance (Xu and Huang, 1995). The analysis of variance and calculation of means was carried out with the SPSS 12.0 statistical program.

3 Results

3.1 Callus induction

Two kinds of plant growth regulator, 2,4-D and 6-BA, were used to select the best callus induction medium. Each of the plant growth regulators were represented by three levels. Abundant light yellow calli could be found from cut pieces of the leaves after being inoculated for 10–15 d (Fig. 1). Callus induction rates and growth status were observed after being inoculated for 25 d. The results show that the effects of 2,4-D, 6-BA and their interaction were all significant (Table 1). The callus induction rate of the medium supplemented with 1.0 mg·L^{-1} 2,4-D is 97.2%, which was markedly higher than that of other treatments. In this medium, the calli grew fast and the texture was friable. The media with both 2,4-D and 6-BA had negative effects on callus induction, in which the calli were more compact and grew at a slower rate. All of the calli became brown after being cultured for more than 35 d. The medium MS + 1.0 mg·L^{-1} 2,4-D + 5 g·L^{-1} agar + 30 g·L^{-1} sucrose was the optimum callus induction medium and the best subculture cycle was 25 d.

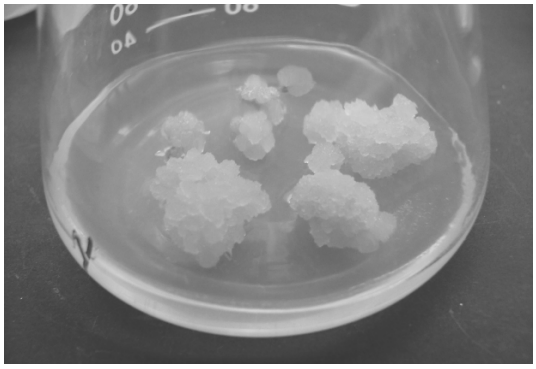

Fig. 1 Calli induced from leaves of *P. tomentosa*

3.2 Establishment of cell suspension line

3.2.1 Selection of initial medium

The calli that were friable and light yellow were inoculated into liquid media supplemented with different concentrations of 2,4-D. Suspension cells were initially generated within 15 d. Morphologically, they largely consisted of cell aggregates of round to oval shaped cells. PCV was surveyed after being cultured on a shaker for 15 d. The results (Table 2) indicate that the yield of cells is the highest for the medium supplemented with 1.5 mg·L^{-1} 2,4-D. The cell aggregates were easy to differentiate adventitious roots after being cultured in a medium with 0.5 mg·L^{-1} 2,4-D for 15 d. When the concentration of 2,4-D was higher than 2.5 mg·L^{-1}, the suspension cells grew so fast that some of them were browning. The medium MS + 1.5 mg·L^{-1} 2,4-D + 30 g·L^{-1} sucrose can be used as the best initial medium for the cell suspension line.

3.2.2 Callus subculture times

Calli which had been subcultured 0, 4 and 8 times were inoculated into the initial medium to initiate cell

Table 1 Effect of plant growth regulators on callus induction of *P. tomentosa*

No.	2,4-D (mg·L^{-1})	6-BA (mg·L^{-1})	Induction rate (%)	Callus status		
				Color	Growth	Texture
1	0.5	0	56.94±3.67 c d	LY	GF	R
2	0.5	0.2	69.45±5.01 b c d	YG	GS	C
3	0.5	0.4	48.61±5.00 c d	YG	GS	C
4	1.0	0	97.22±2.78 a	LY	GF	R
5	1.0	0.2	79.16±4.17 b c	Y	GM	C
6	1.0	0.4	77.78±3.68 b c	Y	GM	C
7	1.5	0	90.28±3.68 a b	DY	GF	R
8	1.5	0.2	38.89±5.01 d	DY	GM	R
9	1.5	0.4	48.61±3.67 c d	DY	GM	R
F	34.256*	28.277*	2,4-D×6-BA: 10.875*			

Notes: * means significant at 0.05 level. LY means light yellow, GF growing fast, R means friable, YG yellow and green, GS growing slowly, C compact, Y yellow, GM growing moderately and DY dark yellow.

suspension culture. Table 3 shows that along with the increase in the number of callus subculture times, the yields of suspension cells are regressive. In addition, browning occurred. The calli that were not subcultured too often, easily yielded fine suspension cells. Moreover, the calli that were not subcultured at all, were the best material for suspension culture.

3.3 Effects of 2,4-D concentration on subculture

Approximately 2.0 g cells of fresh weight were weighed from a stable cell suspension line and transferred into subculture media containing different 2,4-D concentrations as follows: 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg·L^{-1}. Cell growth curves were derived from data obtained by means of PCV values. The error bars indicate that under strict subculture regimes the variations between cultures at any particular age are quite small. Figure 2 indicates cells in a medium, supplemented with 0.8 mg·L^{-1} 2,4-D, grew best. Suspension cells can actively proliferate in this medium (Fig. 3). The yield of the cell line increased like an "S" curve with the changing time of culture. The curves can be divided into three phases: a lagging phase (1–4 d), a logarithmic phase (4–7 d) and a slow phase (8–10 d). The cell line proliferated slowly in the media containing 0.4 mg·L^{-1} 2,4-D or less. The cells grew so fast in the media with high 2,4-D concentrations, that some

Table 2 Effect of concentrations of 2,4-D on establishment of cell suspension line

No.	2,4-D (mg·L^{-1})	PCV (mL)	Cell growth status
1	0.5	3.37±0.35 b	Light yellow, adventitious roots
2	1.0	4.40±0.44 ab	Light yellow
3	1.5	5.40±0.06 a	Light yellow
4	2.0	4.63±0.25 a	Yellow
5	2.5	4.53±0.38 a	Dark yellow, browning

Table 3 Effect of callus subculture times on establishment of cell suspension line

No.	Subculture times	Inoculation callus (g FW)	PCV (mL)	Cell browning status
1	0	2.0	4.20±0.17 a	Naught
2	4	2.0	1.83±0.38 b	A little
3	8	2.0	0.83±0.09 b	Badly browning

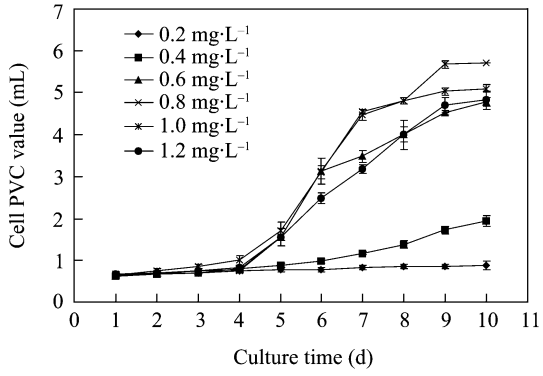

Fig. 2 Growth curves of cell suspension lines in different subculture media

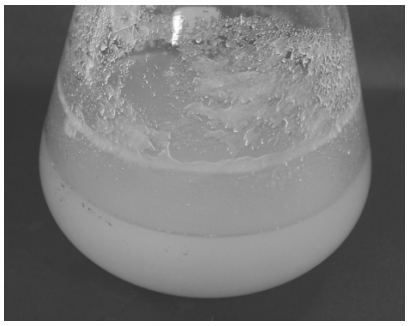

Fig. 3 Stable cell suspension line of *P. tomentosa*

cells became old or died after being cultured for more than 7 d. The medium MS + 0.8 mg·L^{-1} 2,4-D + 30 g·L^{-1} sucrose would be the best medium for cell subculture and the best subculture cycle 7 d.

4 Discussion

The category and concentration of plant growth regulators affect the induction and proliferation of calli. Fine callus can be obtained from *Populus* tissues using a media containing auxin and cytokinin, which had been reported for *P. euphratica* (Liu et al., 2000), *P. tomentosa* (Li and Tian, 2004) and *P. alba*× (*P. davidiana* × *P. simonii*) × *P. tomentosa* (Du et al., 1998). However, in our study, in order to induce callus from leaf tissues of *P. tomentosa*, the addition of 2,4-D is essential. On the other hand, BA, a cytokinin, has a negative effect on callus induction and proliferation. The treatments of BA cause callus to become compact and to grow slowly. The result is similar to the results reported by Wang et al. (1991) about *P. simmonii* and by Lu et al. (1997) for *P. xiaohei*.

In our study, a high level of 2,4-D concentration was needed to initiate cell suspension culture of *P. tomentosa*. However, suspension cells could proliferate in a subculture medium containing a low 2,4-D concentration. The concentration of 2,4-D could be decreased in the subculture in order to reduce the damage to the cells. In the next step of our study, the concentration of 2,4-D in the subculture medium will still be reduced. Cytokinin will be added into the medium in order to promote the regeneration of cells.

The number of subculture times of callus affects the quality of suspension cells. Different plants have their best callus subculture times during the establishment of their cell suspension line. For the calli of potato, subcultured 1–3 times, it was easy to obtain suspension cells (Zhang and Dai, 2000). However, the best material for rice suspension culture was the callus, which had been subcultured 7 times (Yin and Liu, 1994). The result in our study shows that the greater the increase in the number of subculture times, the harder to establish a suspension culture line for *P. tomentosa*. The reason may be that the ability of proliferation usually degrades when the callus is being subcultured too many times. Therefore, in order to obtain a fine cell suspension line, young, friable and coloury calli form the best initial material.

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