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Plants regenerated from mesophyll protoplasts of white mulberry

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ABSTRACT

Morus alba(white mulberry) mesophyll protoplasts were isolated from leaves of 30-45 day old sterile shoots, with protoplast yields of 2.5×10^7 g^{-1}/F.W. after purification. The protoplasts were cultured in a modified K8P liquid medium containing 0.2 mg/L 2,4-D(2,4- Dichlorophenoxy acetic acid), 1 mg/L NAA(Naphthyl acetic acid) and 0.5 mg/L BA(6-benzylaminopurine). A low plating density (5×10^4/ml) proved to be favourable to the division of protoplast-derived cells. The first division occurred 4 days after culture, and the division frequency reached 24% at 10 days. A number of cell colonies and microcalli formed in 6 weeks. The microcalli were transferred onto MSB medium with 0.5 mg/L NAA and 0.5 mg/L BA for further proliferation. Shoot formation was initiated when the calli of 3-4 mm in size were transferred onto MSB differentiation medium with 0.1 mg/L NAA and 1 mg/L BA. The frequency of shoot formation was 35%. The shoots of 4-5 cm in height were excised from the callus and rooted on half strength MS medium with 0.5 mg/L IBA and 0.1 mg/L BA. After transplantation into pots, the regenerated plants grew vigorously in the phytotron.

Key words: *Morus alba L., white mulberry, protoplast culture, plant regeneration.*

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INTRODUCTION

Protoplast culture is one of the most rapidly developing areas in plant tissue culture, because of its importance in plant genetic manipulation. However, so far, there are only a few forest tree species in which plant regeneration from protoplasts has been successful, namely *Liriodendron* tulipifera[1], *Paulownia fortunei*[2], Picea *glauca*[3], *P1atanus orientalis*[4], *Populus sp.*[5-8], *Santalum album*[9], *Solanum dulcamara*[10] and *Ulmus* [1]. White mulberry (*Morus alba*) is an important woody feed crop for the silkworm. Some tissue culture has been carried out with this species, in an attempt to provide artificial feed containing cultured mulberry cells and for clonal propagation (see Review of Oka and Ohyama, [12]). In this paper, we report plant regeneration from mesophyll protoplasts of this species.

MATERIALS AND METHODS

Plant material

White mulberry seeds, collected in our institute, were surface-sterilized by dipping in 70% alcohol for 30 sec and then soaking in a sodium hypochlorite solution (containing 0.5% of effective chlorine) for 15 min. After being washed with sterile water (4 changes), the sterilized seeds germinated on hormone-free MS medium[13]. Apical buds cut from the seedlings were cultured on MS medium supplemented with 0.2 mg/L BA (at 25 ℃; 2000 Lux, 12 h/day) for inducing formation of multiple shoots. The multiple shoots formed were subcultured once a month for micropropagation of the plant material.

Protoplast isolation and culture

Young expanded leaves, collected from 30-45 day old cultured shoots, were cut into 0.5-1 mm strips and plasmolyzed for 1.5-2 h in CPW-11M (containing 11% mannitol). For releasing protoplasts, the leaf strips were digested statically in an enzyme solution for 4 h and then on a shaker (35 rpm) for 1 h. The enzyme solution was consisted of 1% Cellulase Onozuka R-10, Macerozyme R-10 and 0.05% Pectoltase Y-23 in CPW-9M (containing 9% mannitol), pH 5.7. The protoplasts in enzyme solution were filtered through an 45 μm nylon sieve and collected by centrifuging at 800 rpm for 4 min. They were purified by using a routine procedure employing floatation in CPW-18S (containing 18% sucrose) and 3 washes in CPW-9M solution (centrifuged at 1000 rpm for 3 min). The purified mesophyll protoplasts were counted and protoplast viability was examined with 0.1% FDA in 0.35 mol/L glucose solution.

The mesophyll protoplasts were cultured in 6 cm plastic dishes, each containing 2 ml K8P liquid medium[14], supplemented with 0.2 mg/L 2,4-D, 1 mg/L NAA and 0.5 mg/L BA, at densities of 1×10^4, 5×10^4, 1×10^5, 5×10^5, 1×10^6 and 5×10^6 protoplasts/ml to compare the effect of plating density on the division of the protoplast-derived cells. The dishes were placed at 25±1℃ in dark. Division frequency was counted at 10 day. After small colonies formed (about 10 d), the dishes were moved under light (about 500 lux), and the cultures were diluted at 10 d intervals with 0.5 ml K8 medium[14] with 0.1 mg/L 2,4-D, 1 mg/L NAA and 0.2 mg/L BA, for sustaining cell divisions and further stimulating cell colony formation and growth. Plating efficiency (% of number of small calli and cell colonies formed in the total number of the protoplasts cultured) was counted after 5-6 weeks of culture when small calli of 0.5-1.0 mm in size formed.

Callus proliferation

The small calli of 0.5-1.0 mm in size were transferred onto MSB growth medium (containing MS

minerals[13] and B_5 organic components[15]) supplemented with 0.1-0.5 mg/L NAA and 0.25-1.0 mg/L BA for further growth and proliferation. The culture conditions were 25 ± 1℃ with a 12 h photoperiod at 1000 Lux.

Plant regeneration

After 2 weeks on MSB growth medium, the calli grew up to 3-4 mm in size. Compact and light yellow calli were picked out and transferred onto MSB differentiation media with different types and concentrations of hormones for shoot formation. Shoots of 4-5 cm in height were cut off and rooted on half strength MS medium with 0.5 mg/L IBA and 0.1 mg/L BA. Regenerated plants were transplanted into pots, and grown in the phytotron of our institute.

RESULTS and DISCUSSION

Mulberry protoplasts were readily isolated from young expanded leaves of sterile shoots (Fig 1, 2), with a high yield of 2.5 $\times 10^7$g/F.W. after purification. FDA fluorescent staining showed that the viability of isolated protoplasts reached 96.8%. Pretreatment of the leaf strips in CPW-11M for plasmolysis reduced the time required for enzymatic digestion. Incubation in enzyme solution, firstly in stationary and then on a shaker, caused less damage to protoplasts and was beneficial to maintain protoplast survival.

The data listed in Tab 1 showed that protoplast density in culture apparently affected the divisions of the protoplast-derived cells. Within range of 1×10^4-5×10^6 /ml, the best results of time needed to initiate the first division and division frequency was from the group of 5×10^4 /ml protoplast density. In this case, the first division was observed at 4 d (Fig 3), the second division at 7 d (Fig 4), with cell clusters at 12 d (Fig 5). Higher densities (5×10^5, 1×10^6, 5×10^6) were unsuitable for the initiation of division and its maintenance for colony formation. It was demonstrated that mulberry mesophyll protoplasts could be induced to divide and form colonies when they were cultured in a thin layer of liquid medium at lower density. A number of small calli of 0.5-1.0 mm in size formed after 6 weeks of culture (Fig 6).

Tab 1. Effect of protoplast densities on division frequency

Protoplast density (per ml)	1st division (days)	2nd division (days)	Colony formation (days)	Division frequency at 10 days (%)
1×10^4	6	8	14	18.8
5×10^4	4	8	12	24.0
1×10^5	7	9	16	16.4
5×10^5	7	9	18	15.4
1×10^6	10	12	19	7.2
5×10^6	10	14	21	6.5

Hormone components in both the proliferation medium and the differentiation medium remarkably affected shoot regeneration from protoplast-derived calli (Tab 2 and 3). When the small calli of 0.5-1.0 mm were transferred onto MSB proliferation medium with 0.25-0.5 mg/L NAA and 0.25-1 mg/L BA, compact and light-yellow calli with a potential for shoot formation were formed (Fig 7)., From the data in Tab 2, the best results in shoot regeneration was obtained in the calli cultured on the proliferation medium with 0.5 mg/L NAA and 0.5 mg/L BA. In order to test the effect of hormone component and concentration in differentiation medium on shoot formation, the calli of 3-4 mm in size cultured on the proliferation medium with 0.5 mg/L NAA and 0.5 mg/L BA were transferred onto MSB media supplemented with 0.1 mg/L NAA and different concentrations of BA or ZT (zeatin). The data in Tab 3 show that the differentiation medium with 0.1 mg/L NAA and 1 mg/L

Tab 2. Effects of hormone components in proliferation medium on shoot regeneration from mesophyll protoplast-derived calli of muiberry

Proliferation medium* (mg/L)	Differentiation medium* (mg/L)	No. of inoculated calli	No. of calli with producing shoots	Frequency of shoot formation(%)
NAA 0.1+BA 1.0		100	0	0
NAA 0.25+BA 0.25		120	3	2.5
NAA 0.25+BA 0.5		125	5	4.0
NAA 0.25+BA 1.0	NAA 0.1+BA 1.0	120	7	5.8
NAA 0.5+BA 0.25		120	16	13.3
NAA 0.5+BA 0.5		128	45	35.1
NAA 0.5+BA 1.0		120	24	20.0

* Basic medium: MSB-MS minerals+B_5 organic components.

Tab 3. Effects of hormone components in differentiation medium on shoot regeneration from mesophyll protoplast-derived calli of mulberry

Proliferation medium* (mg/L)	Differentiation medium* (mg/L)	No. of inoculated calli	No. of calli with producing shoots	Frequency of shoot formation(%)
NAA 0.5+BA 0.5	NAA 0.1+BA 0.5	86	18	20.9
	NAA 0.1+BA 1.0	128	45	35.1
	NAA 0.1+BA 2.0	114	29	25.4
	NAA 0.1+ZT 1.0	90	14	15.6
	NAA 0.1+BA 0.5+ ZT 0.5	102	24	23.5

* Basic medium: MSB-MS mineral+B_5 organic components.

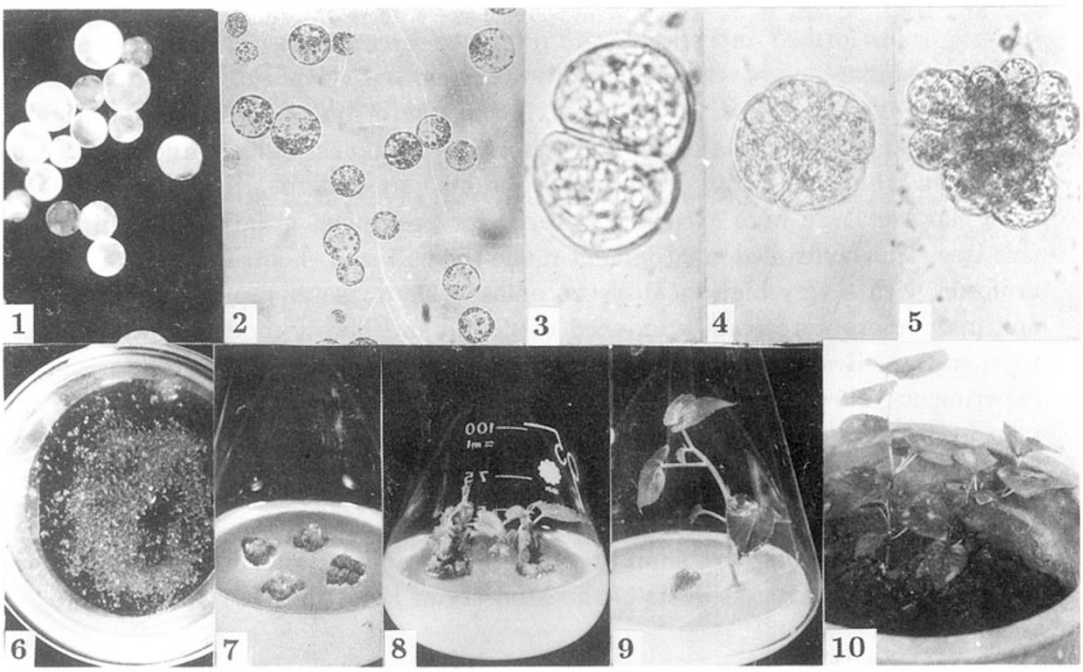

Figure Explanation

Fig 1-10. Protoplast culture and plant regeneration of white mulberry.

Fig 1. Viability of freshly isolated mesophyll protoplasts of white mulberry was examined with FDA (fluorescein diacetate) staining. Viable protoplasts made up 96.8% of the population. × 400

Fig 2. Freshly isolated mesophyll protoplasts from sterile shoots. × 400

Fig 3. The first division occurred after 4 d of culture. × 400

Fig 4. The second division occurred after 7 d of culture. × 400

Fig 5. Cell colony formed at 12 d in protoplast culture, × 100

Fig 6. Protoplast-derived calli in 60 × 15 mm plastic Petri dish, after 6 weeks of culture in K8p liquid medium with 0.2 mg/L 2, 4-D, 1 mg/L NAA and 0.5 mg/L BA.

Fig 7. Compact and light-yellow calli formed on MSB proliferation medium with 0.5 mg/L NAA and 0.5 mg/L BA.

Fig 8. Shoot formation on MSB differentiation medium with 0.1 mg/L NAA and 1 mg/L BA.

Fig 9. Complete plant regenerated after rooting on half strength MS medium with 0.5 mg/L IBA and 0.1 mg/L BA.

Fig 10. The regenerated mulberry plant grown in pot.

BA gave the best result. Green spots appeared on the calli 2-3 weeks after transfer onto this medium, followed by bud formation through organogenesis (Fig 8). The frequency of shoot formation reached 35%. When green shoots of 4-5 cm in height were cut off and transferred onto half strength MS rooting medium, vigorously growing roots formed and plants were produced 2 weeks later (Fig 9). Some of the regenerated plants were transplanted into pots, with survival rate of more than 90%. They grew into trees with 10-14 leaves, in the Phytotron of our institute (Fig 10).

To our knowledge, this is the first report on protoplast culture and plant regeneration in white mulberry. The results obtained showed that the expanded leaves from shoot culture were excellent material for protoplast isolation and culture for this tree. The controlled environment made the cultured shoots easily available and uniform, with a very high yield of protoplast. The mesophyll protoplasts from the cultured shoots were readily induced to divide and had a good potential of plant regeneration. Besides, the procedure we developed also gave high reproducibility in experiments. Now it is possible to improve this important tree species through gene transfer by using protoplasts as recipient.

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