Micropropagation of Mulberry (*Morus alba* L.) by Liquid-Shake Culture of Multiple-Bud Bodies

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INTRODUCTION

Mulberry is an important woody crop used for silkworm rearing and is cultivated in many temperate countries throughout the world. It is conventionally propagated by grafting or cuttings. In vitro micropropagation of mulberry has been studied for various species (Oka, 1985; Hossain et al., 1992; Jain et al., 1990; Yadav et al., 1990). In these studies, micropropagation through multiple shoot formation has been exclusively performed on solid media. Micropropagation by liquid-shake culture is employed in several vegetables and ornamental flowers (Takayama, 1991). In woody plants, however, only a limited number of studies have reported on micropropagation using liquid culture (Alvard et al., 1993; Hammerschlag, 1982). In preliminary work, we reported that multiple-bud bodies (MBB) could be induced from shoot tips of mulberry seedlings immediately after development of the first true leaves in the liquid-shake culture of a medium supplemented with CPPU, a urea-type cytokinin (Hayashi and Oka, 1995). In this report, the same culture system was applied to a mulberry cultivar commercially cultivated in Japan to obtain MBB with higher multiplication rates than shoot cultures in the conventional culture system using solid media.

MATERIALS AND METHODS

Initiation of Shoot Cultures. Winter buds of mulberry 'Kenmochi' were sterilized with sodium hypochlorite solution (1% effective chlorine) for 30 min. After rinsing three times with sterile water, meristems (3 to 5 mm long) were dissected from the winter buds after removing scale leaves, and cultured on Murashige and Skoog (1962) (MS) medium containing 3% fructose and 1 mg liter^{-1} benzyladenine (BA). The medium was solidified with 0.8% agar. The pH of the medium was adjusted to 6.0 before autoclaving at 120C for 15 min. After 1 month of culture at 25C under a 19-h photoperiod, developed shoots were transferred to a fresh medium with the same components as the shoot initiation medium. Shoot cultures thus established and subcultured monthly were employed as materials for further experiments.

Induction and Subculture of MBB. Shoot tips (1 to 2 mm) taken from in vitro-grown shoots were cultured in liquid MS medium containing 3% sucrose and BA or N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) (Fulmet: Kyowa Hakkou Kogyo). Explants were cultured either in Erlenmeyer flasks (100 ml) containing 30 ml liquid medium which were rotated horizontally at 120 rpm, or in tubes (24 mm × 120 mm) containing 10 ml medium which were placed on a drum rotating vertically at 2 rpm. The cultures were maintained at 25 C under a 19-h photoperiod with diffuse fluorescent light. MBB divided into small pieces including several buds were subcultured for maintaining stock cultures every 2 weeks in MS liquid medium with 3% sucrose and 1 mg liter^{-1} CPPU (pH 5.8).

Table 1. Multiple bud body (MBB) induction from shoot tips of mulberry (cv. Kenmochi). Shoot tips were cultured in flasks rotated horizontally (120 rpm) for 4 weeks.

Cytokinin (mg liter^{-1})	Percentage of explant with: Leaf development	Shoot elongation	MBB formation	No growth
BA[1] (1)	8	92	0	0
BA (2)	75	25	0	0
BA (5)	100	0	0	0
BA (10)	0	0	0	100
CPPU (2)	0	0	83	17

[1] Abbreviations: BA, benzyladenine; CPPU, N-(2-chloro-4-pyridyl)-N-phenylurea.

Table 2. Effects of rotation methods on multiple bud body (MBB) formation (cv. Kenmochi).

Rotation method	% MBB formation: 2 weeks	4 weeks	6 weeks	8 weeks	No. of buds per MBB after 8 weeks culture ±s.d.
Vertical (2 rpm)	0	0	46	58	10. 8±3. 9
Horizontal (120 rpm)	0	100	100	100	33.1±6.1

Table 3. Proliferation of a single bud excised from MBB (cv. Kenmochi) cultured for 3 weeks in medium with BA, TDZ, and CPPU[1].

Cytokinin (mg liter^{-1})	% explant with: Leaf development	Shoot elongation	MBB formation	No. buds/MBB ±s.d.
CPPU (2)	0	0	100	12.3±2.6
CPPU (5)	0	0	93	6.9±2.8
TDZ (2)	13	0	87	6.2±1.8
TDZ (5)	3	0	93	6.8±2.6
BA (2)	20	73	7	8.5±1.2
BA (5)	77	13	10	4.3

[1] Abbreviations: BA, benzyladenine; CPPU, N-(2-chloro-4-pyridyl)-N-phenylurea; TDZ, thidiazuron.

Plant Regeneration from MBB. Shoots were regenerated on MS solid medium (0.8% agar) containing 3% fructose and 1 mg liter^{-1} BA (pH 6.0) from bud explants excised from subcultured MBB. The regenerated shoots were transferred to MS medium with 0.1 mg liter^{-1} α-naphthaleneacetic acid (NAA) for root induction.

RESULTS AND DISCUSSION

MBB were formed from shoot tips when they were cultured in a medium with 2 mg liter^{-1} CPPU (Table 1). MBB were occasionally associated with small leaves but shoots never elongated while rotary culture was continued (Fig. 1). BA induced shoot elongation at 1 mg liter^{-1} and leaf development at 5 mg liter^{-1} from the shoot tips, but was ineffective for MBB formation even when its concentration was elevated to 10 mg liter^{-1} (Table 1). The optimum concentration of CPPU for inducing MBB was 2 mg liter^{-1}. Shoot tip explants initially grew very slowly, inducing MBB 4 to 6 weeks after culture initiation. Horizontal rotation (120 rpm) of the medium in flasks induced MBB more rapidly with a larger number of buds per MBB than vertical rotation (2 rpm) of the medium in tubes (Table 2); hence the following experiments were conducted using the horizontal rotation culture method. When a single bud excised from MBB was subcultured, its responses to cytokinins were different (Table 3). CPPU and thidiazuron (TDZ) regenerated MBB at higher rates than BA. On the other hand, BA stimulated leaf or shoot growth rather than MBB regeneration as was the case of the initial culture of the shoot tips. The multiplication rates of single buds excised from MBB on medium containing CPPU were quite stable, ranging from 16 to 20 per 4 weeks during three consecutive subcultures. When a single bud was transferred to MS medium containing either CPPU or BA at 1 mg liter^{-1}, shoot elongation occurred 14 to 21 days after transfer to the stationary culture conditions. Most of the regenerated shoots exhibited a substantially normal appearance with negligible vitrification. Over 50% of the explants cultured on medium supplemented with 0.1 to 1.0 mg liter^{-1} CPPU formed roots following shoot regeneration, while rooting frequency in the presence of BA was less than 20%. Furthermore, normal rooting from excised shoots was observed when they were cultured on a medium with 0.1 mg liter^{-1} NAA. The present study revealed that CPPU was specifically effective for inducing MBB in mulberry. Once MBB had been induced, subsequent subculture of MBB and shoot regeneration were so easy and stable that propagation using MBB showed promise, particularly in such cultivars as 'Kenmochi', in which the multi-

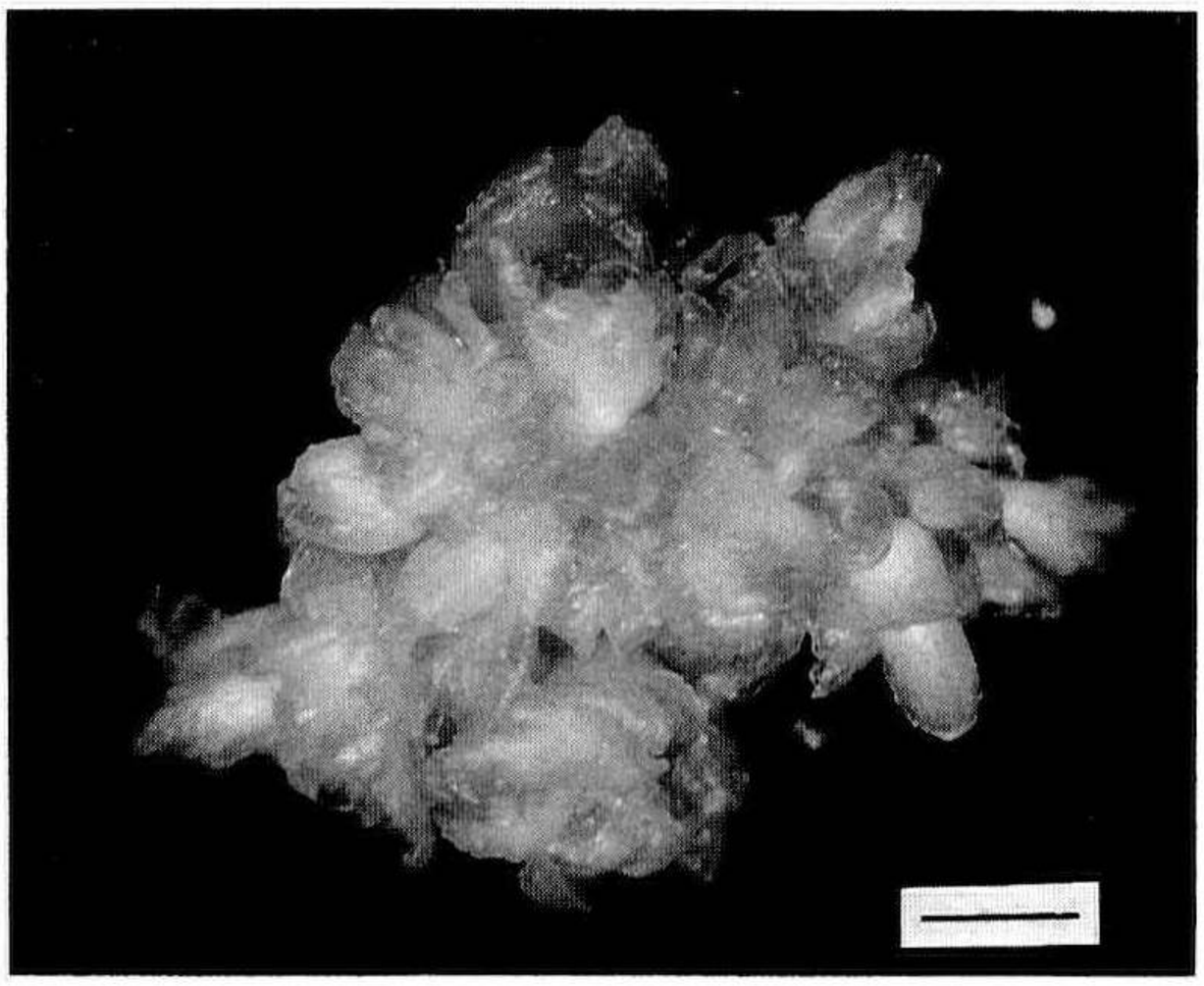

Figure 1. Multiple-bud body (MBB) of mulberry (cv. Kenmochi) induced from a shoot tip by liquid shake culture on MS medium supplemented with 2 mg liter^{-1} CPPU (N-(2-chloro-4-pyridyl)-N-phenylurea) (scale bar is 2 mm).

plication rate was remarkably increased compared to a rate of 3 to 4 per month by the conventional solid medium propagation system (Oka, 1985). TDZ, another urea-type cytokinin, shows stronger cytokinin effects such as more vigorous shoot proliferation than BA in many woody plant materials (Huetteman and Preece, 1993). In mulberry, however, CPPU was more effective than TDZ for inducing MBB, as shown in a previous study (Hayashi and Oka, 1995), indicating that the mode of action between the two cytokinins is different in this species.

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