Preservation of Genetic Resources of Mulberry by Means of Tissue Culture

By Sueo Enomoto

Department of Cell Biology, National Institute of Agrobiological Resources
(Yatabe, Ibaraki, 305 Japan)

Many basic studies have so far been carried out on *in vitro* culture of isolated buds of mulberry[5-10]. At present, it is possible to culture aseptically-isolated winter buds, axillary buds, or shoot tips in the test-tube. Even the example of growing up perfect whole plants was reported[6].

The technique of *in vitro* culture of isolated buds of mulberry is applicable to the mass-propagation in the test-tube, or germplasm preservation in the test-tube. To preserve germplasm of mulberry, it has been the practice to grow a large number of plants in the fields from year to year. This practice requires a lot of labor for cultural management and a large scale of the field. This has become a serious problem in the germplasm preservation. On the other hand, the present situation is that the introduction and preservation of much more genetic resources are required in order to develop more excellent varieties of mulberry.

Therefore, the author attempted to establish an efficient method of germplasm preservation of mulberry by making use of the *in vitro* culture of isolated buds. A series of experiments were conducted using Shimaguwa (*Morus acidosa* Griff) and other materials introduced from foreign countries. As a result, it was confirmed that the *in vitro* culture of isolated buds was extremely useful for the preservation of mulberry plants as the genetic resources[1,2]. The result is briefly presented in this paper.

Materials and experimental methods

The materials used for the present study totaled 24 strains given in Tables 4–1 and 4–2. They are composed of four strains of Shimaguwa and 20 strains of foreign varieties. The isolated buds used for the experiment were taken from plants growing in the varietal preservation field or from potted seedlings growing in a greenhouse in the Sericultural Experiment Station, Ministry of Agriculture, Forestry and Fisheries.

The isolated buds to be used were taken from axillary buds or apical buds. At first, axillary buds were removed from plants in the form of "one bud with one internode", and apical buds were removed as "blocks of core tissue of 1–1.5 cm in length" from the base of unfolded leaves.

After immersed in 70% ethanol for 5 sec., they were immersed in antiformin solution (10% antiformin solution (v/v) was diluted 10 times) for 30 min with stirring (using a stirrer). After the sterilization, the materials were washed 3 times with sterilized water.

Then, leafblades and stipules of unfolded leaves of axillary buds and apical buds, thus prepared, were removed with a scalpel, and the core tissues of 4–5 mm in length, including meristem, were isolated as explants. Each of them was transplanted onto the culture medium in each test-tube.

Table 1. Organ culture experiments so far conducted in Japan for mulberry

Species	Explant	Culture medium	Reference
Morus alba	Nodes	MS	Oka and Ohyama (1975)[9]
	Leaf	MS	Oka and Ohyama (1981)[10]
	Hypocotyl	MS	Ohyama and Oka (1982)[7]
M. bombycis	Winter-bud	MS	Oka and Ohyama (1974)[8]
M. kagayama	Shoot-tip	MS	Ohyama and Kawakita (1975)[5]
M. indica	Nodes	MS	Patel et al. (1983)[11]
	Nodes	MS	Mhattre et al. (1985)[3]

Composition of culture medium

Composition of culture medium was examined to know the medium suitable for the isolated buds. Growth of the isolated buds varied with a kind of sugar and growth regulator, showing the characteristic nutrient requirement. Here, the modified MS medium, which is better adapted to the isolated buds of mulberry will be described.

1) Inorganic salts: The culture medium which has so far been used for various kinds of isolated buds of mulberry was the MS medium (Murashige and Skoog medium[4]) as shown in Table 1. In the present study, the modified MS medium (Table 2) gave better growth.

2) Carbon source: For tissue culture, sucrose or glucose is often used. However, it was made clear that 3% fructose was most effective in promoting shoot growth in an initial culture of isolated buds (Fig. 1). However, the isolated buds, which had grown to a certain level, were able to continue their growth with sucrose.

3) Growth regulator: For the growth of isolated buds of mulberry, some growth regulators were required, particularly the supply of cytokinin was essential. Then, different effectiveness of different kinds of cytokinin

Table 2. Constituents of the Murashige and Skoog basal medium (partly modified)

	mg/l		mg/l
KNO_3	1,900	KI	0.83
NH_4NO_3	1,650	$Na_2MoO_4 \cdot 2H_2O$	0.25
$CaCl_2 \cdot 2H_2O$	440	$CuSO_4 \cdot 5H_2O$	0.025
$MgSO_4 \cdot 7H_2O$	370	$CoCl_2 \cdot 6H_2O$	0.025
KH_2PO_4	170	Inositol	100
Na-Fe-EDTA	32	Glycin	2
H_3BO_3	6.2	Thiamine·HCl	0.1
$MnSO_4 \cdot 4H_2O$	22.3	Nicotinic acid	0.5
$ZnSO_4 \cdot 4H_2O$	8.6	Pyridoxin·HCl	0.5

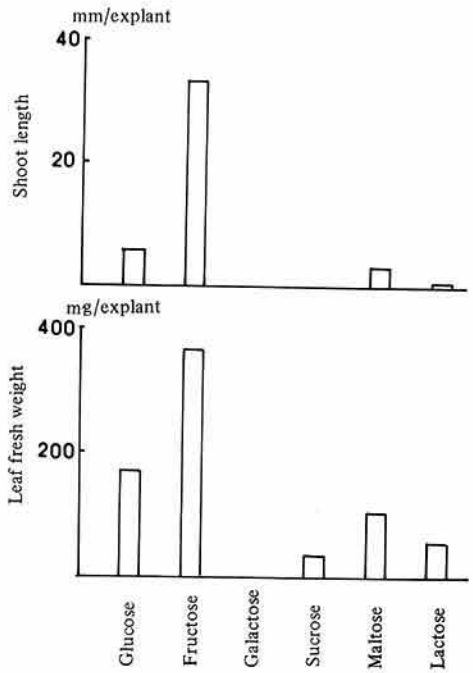

Fig. 1. Effect of sugar on shoot development from winter buds (Oka)[7]
Culture media containing sugar were autoclaved.
Variety: Ichinose.
Culture period: 35 days.

Table 3. Effect of cytokinin on shoot development from winter buds (Oka)[7]

Cytokinin		Leaf fresh weight	Shoot length
	mg/*l*	mg/explant	mm
Kinetin	0.01	0	0
	0.1	0	0
	1	0	0
	10	0	0
	100	65.1	5.5
BA	0.01	0	0
	0.1	356.9	15.8
	1	379.5	23.8
	10	0	0
Zeatin	0.1	103.4	0.3
	1	248.5	15.5

Variety: Kenmochi. Culture period: 30 days.

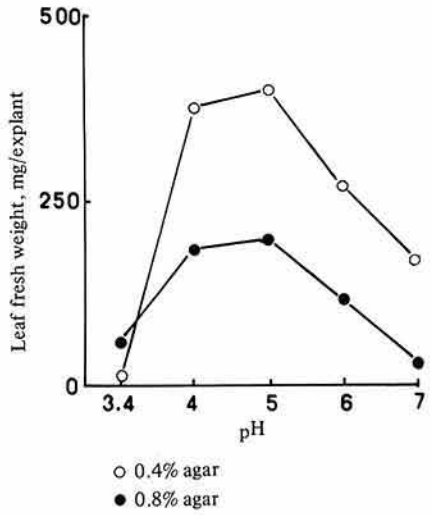

Fig. 2. Effect of pH and agar concentration of culture media on leaf development of isolated winter buds (Oka)[7]
Medium: MS basal medium+1.0 mg/*l* of BA+3% sucrose.
Variety: Kenmochi.
Culture period: 30 days.

and their necessary dosage were examined. As given in Table 3, the addition of 6-benzyladenine (BA) at the rate of 1 mg/*l* gave the best result.

4) Agar concentration and pH: Agar concentration and pH of the basal MS medium influenced bud growth. Of 4% and 8% of the agar concentration, the former showed better growth, and pH 5 seemed optimal (Fig. 2). However, the use of fructose as the carbon source made the culture medium somewhat soft after autoclaving, so that the transplanted buds are liable to sink into the medium. Therefore, agar concentration of 8% and pH 5.6 was adopted.

Preparation of culture media and cultural condition

The test-tube used was 24ϕ × 120 mm in size. After receiving 20 m*l* of the modified MS medium, the tube was stoppered with alminium foil and autoclaved at 121°C for 10 min for sterilization. The 10-min period for autoclaving was adopted in order to prevent quality change of fructose in the medium.

The cultural condition was 27° ± 1°C and 12 hr of illumination (ca. 4,000 lux) by fluorescent lamps per day.

Growth of isolated buds

The isolated buds started to expand few days after the beginning of the culture, and leaf unfolding was recognized on the 7–10th day. When growth advanced further, shoots began to elongate. The growth on the 30th day of the culture was examined. The result was as follows:

Contamination of isolated buds with bacteria and fungi occurred in a part of the culture. Excluding the contaminated buds, all isolated buds were classified into 3 growth levels, i.e., A: shoot developed (Plate 1), B: only leaf growth, and C: almost no growth. Tables 4–1 and 4–2 show the result.

Some difference in growth was observed among strains. Of 4 strains of Shimaguwa, Hoshino showed the highest proportion of A, 86.4%, as compared with other strains showing about 60%. M-32 and Yakushima showed higher proportion of C, about 35%, in contrast to 0% in Hoshino. The number of shoots developed per bud at the A level was 5 in Hoshino as compared with 2–4 in other strains.

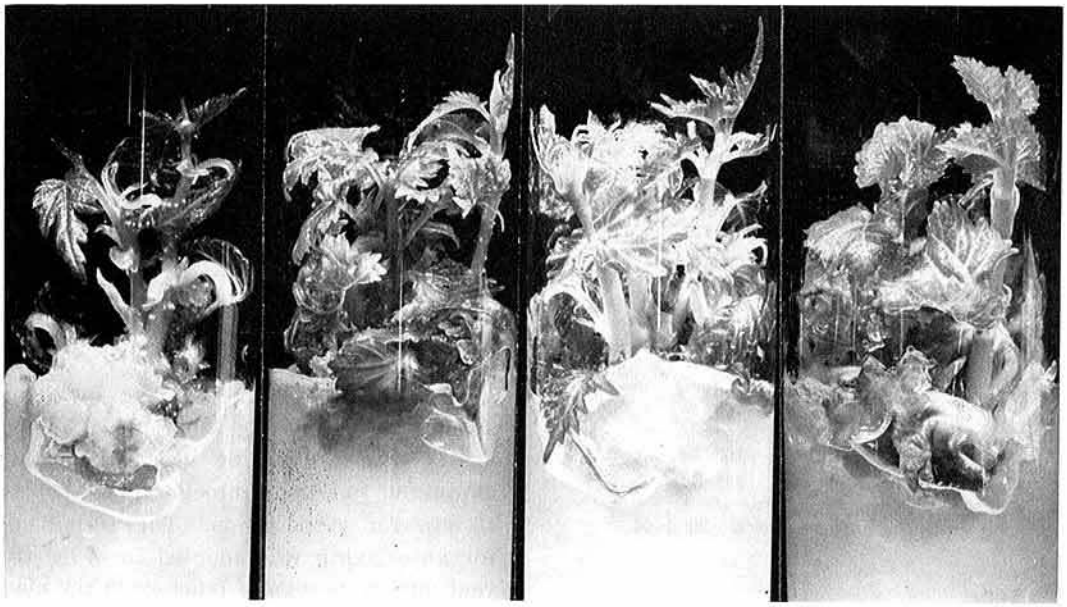

Plate 1. *In vitro* growth of isolated buds of foreign mulberry
Culture period: 30 days.

Among the foreign varieties tested, KAI–2 and KAI–4 of India, 1–9 and 1–12 of Pakistan, Keaw of Thailand, and a Paraguay strain showed higher proportion of the A level growth. The lowest proportion (16.7%) of A was shown by Canada 1, while all other varieties showed A higher than 33.3%. The number of shoots (average) developed per A-bud also showed varietal differences: Som and Keaw of Thailand showed the greatest number, 6.1 and 5.7 respectively. In addition, Sulawesi of Indonesia, Poo of Thailand, Berhompore of India showed the moderately large number, about 3 (Plate 1).

Thus, it was possible to grow isolated buds in the test-tube for all of 24 strains tested. In addition, many of the buds classified as B at the date of examination proceeded later to the A level.

Table 4–1. *In vitro* growth of isolated buds of *Morus acidosa* Griff

Strain	Explant (bud)	Growth of isolated buds			No. of shoots/ explant
		A(%)	B(%)	C(%)	
M–32	Axillary	64.3	0	35.7	2
Yakushima		63.2	0	36.8	3
Hoshino		86.4	13.6	0	5
Inoda		68.4	15.8	15.8	4

A: Vigorous growth of shoots. B: Only leaf growth. C: No growth.
Culture period: 30 days.

Table 4-2. *In vitro* growth of isolated buds of foreign mulberry

	Strian	Explant (bud)	Growth of isolated buds			No. of shoots/ explant
			A(%)	B(%)	C(%)	
India	KAI-2	Shoot tip	95	5	0	1.7
	KAI-4		100	0	0	—
	KAI-6		60	40	0	1.7
	KAI-9		40	60	0	2
	Berhompore	Axillary tip	36.4	63.6	0	3.1
Pakistan	1-9	Shoot tip	81	19	0	1.8
	1-12		86.4	9.1	4.5	1.9
	1-14		66.4	33.3	0	1.7
Thailand	Som	Axillary bud	35	15	50	6.1
	Poo		36.8	63.2	0	3.3
	Dapang		60.9	30.4	8.7	2.1
	Keaw		87	4.3	8.7	5.7
Indonesia	*M. latifolia*		71.4	23.8	4.8	2.3
	Sulawesi		73.7	10.5	15.8	3.7
Canada	1	Shoot tip	16.7	55.6	27.7	1.7
	2		33.3	66.7	0	1
Turkey	T-1		57.1	14.3	28.6	2
	T-2		69.2	15.4	15.4	1.2
Lebanon			45	25	30	1
Paraguay			88.9	11.1	0	2.1

A: Vigorous growth of shoots. B: Only leaf growth. C: No growth.
Culture period: 30 days.

Passage culture of isolated buds

To continue passage culture of isolated buds in the test-tube, it is necessary to examine some points of the cultural condition.

Firstly, fructose added to the MS medium as a carbon source at the initial stage of the culture can be replaced by sucrose, which is sufficient to promote shoot elongation at the later stages.

The growth regulating substance, BA, gave different effect according to different rates of application. The rate of 1 mg/*l* induced an increased number of shoots due to axillary bud elongation. However, in some strains like Som and Poo of Thailand, callus formation or tissue swelling occurred at the basal portion of shoots, which retarded shoot growth. At the rate of 0.1 mg/*l* of BA, all the strains used showed normal growth of isolated buds, which produced roots later and became perfect plant body in the test-tube. On the BA-free culture media, rooting occurred about 10 days after transplanting with all strains of mulberry and perfect plants were developed. In this case, the number of shoots did not increase in many strains.

For the preservation of germplasm of mulberry in the test-tube, the hormone-free condition was suitable. To preserve a mulberry plant, using a test-tube of $20\phi \times 120$ mm in size, the culture is renewed at an interval of about 3–5 months.

Multiplication and acclimatization of shoots

By transplanting axillary buds or apical buds themselves directly onto the MS medium containing 1 mg/*l* of BA, shoots can be obtained. Then, they are transferred to the hormone-free medium to induce rooting. Thus, they become perfect plants (Plate 2). The well-rooted individuals were selected, and after washing the roots thoroughly to remove the culture medium, they were transplanted to pots containing peat moss, etc., to acclimatize them. After the acclimatization, they

Plate 2. Root initiation from shoot tips growing on the hormon-free culture medium
Variety: *Morus acidosa* Griff.
Culture period: 30 days.

were successfully transferred to the field.

Conclusion

It was confirmed that the preservation of mulberry plants in the test-tube by means of the tissue culture method was possible for all 24 strains consisting of 4 Shimaguwa and 20 foreign mulberry strains examined. The *in vitro* growth of isolated buds showed some differences among the strains.

Mass multiplication of shoots, acclimatization of shoots, and transplanting of the shoots grown in the test-tube to the field were experimentally carried out with success. However, further improvement is needed to establish a practical system of germplasm preservation by means of *in vitro* culture of isolated buds of mulberry.

The preservation of mulberry germplasm by the passage culture is considered to have a wide range of applicability for the supply of materials to the future biotechnological works.

References

1) Enomoto, S., Oka, S. & Ohyama, K.: In vitro culture of isolated buds of *Morus acidosa* Griff. *J. Sericul. Sci. Jpn.*, 54, 167–168 (1985) [In Japanese].
2) Enomoto, S. & Ohyama, K.: In vitro culture of isolated buds of foreign mulberry. *J. Sericul. Sci. Jpn.*, 55, 87–88 (1986) [In Japanese].
3) Mhattre, M., Bapat, V. A. & Rao, P. S.: Regeneration of plants from the culture of leaves and axillary buds in mulberry (*Morus indica* L.). *Pl. Cell Rep.*, 4, 78–80 (1985).
4) Murashige, T. & Skoog, A.: A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant.*, **15**, 473–497 (1962).
5) Ohyama, K. & Kawakita, H.: In vitro culture of shoot tips infected with mulberry dwarf. *J. Sericul. Sci. Jpn.*, 44, 413–414 (1975) [In Japanese].
6) Ohyama, K. & Oka, S.: Regeneration of whole plants isolated from shoot tips of mulberry tree. *J. Sericul. Sci. Jpn.*, 45, 115–120 (1976) [In Japanese with English summary].
7) Oka, S.: In vitro culture of isolated bud and organ formation in mulberry. *Bull. Sericul. Exp. Sta.*, 29, 747-852 (1985) [In Japanese with English summary].
8) Ohyama, K. & Oka, S.: Multiple shoot formation from mulberry (*Morus alba* L.), hypocotyls by 2-chloro, 4-pyridyl, N-phenylurea. *In* Proc. 5th int. cong. plant tissue and cell culture, 149–150 (1982).
9) Oka, S. & Ohyama, K.: Studies on in vitro culture of excised buds in mulberry tree. I. Effects of growth substances on the development of shoots and organ formation from winter buds. *J. Sericul. Sci. Jpn.*, 43, 230–235 (1974) [In Japanese with English summary].
10) Oka, S. & Ohyama, K.: Studies on in vitro culture of excised buds in mulberry tree. II. Effects of growth substances on the development of shoots from axillary bud. *J. Sericul. Sci. Jpn.*, 44, 444–450 (1975) [In Japanese with English summary].
11) Oka, S. & Ohyama, K.: In vitro initiation of adventious buds and its modification by concentration of benzyladenine in leaf tissues of mulberry (*Morus alba*). *Can. J. Bot.*, **59**, 68–74 (1981).
12) Patel, G. K., Bapat, V. A. & Rao, P. S.: In vitro culture of organ explants of *Morus indica*, plant regeneration and fruit formation in axillary bud culture. *Z. Pflanzenphysiol. Bd.*, 111, S, 465–468 (1983).

(Received for publication, October 2, 1961)