JARQ 33, 267–274 (1999)

Effects of Medium Conditions on Adventitious Bud Formation in Immature Mulberry Leaves

Hiroaki YAMANOUCHI, Akio KOYAMA and Hiroaki MACHII

Department of Sericulture, National Institute of Sericultural and Entomological Science (Tsukuba, Ibaraki, 305-8634 Japan)

Abstract

Immature mulberry leaves isolated from winter buds were cultured on MS media7) differing in the nitrogen source, sugar(s) or pH, or containing various kinds and concentrations of plant hormones, antibiotics or a herbicide. The ratio of the concentration of nitrate ion to that of ammonium ion remarkably affected the frequency of adventitious bud formation (FABF) and ratios in the ranges of 1:1 and 3:1 were optimum. The most suitable sugars for adventitious bud formation were sucrose, glucose and fructose among the tested sugars. The highest FABF was usually observed in the medium containing 1 μM thidiazuron and the addition of 1 μM abscisic acid further enhanced FABF. Changes of pH and addition of other plant hormones did not produce better results than in the control. Kanamycin, geneticin, hygromycin and bialaphos suppressed adventitious bud formation, showing that the antibiotics and the herbicide may be effective for the selection of transgenic plantlets of mulberry.

Discipline: Biotechnology
Additional key words: nitrogen source, sugar, thidiazuron, abscisic acid, antibiotic

Introduction

Stable regeneration systems of crops are important for the application of more advanced techniques, i.e. genetic transformation by introduction of foreign gene(s). First successful adventitious bud formation of mulberry was reported by Oka and Ohyama[11], who were able to induce adventitious buds on leaves removed from shoots cultured *in vitro*. On the basis of the first report, a more convenient single-step adventitious bud induction method was developed, whereby adventitious buds were formed on cultured immature leaves isolated from winter buds of field-grown mulberry[13]. It is also possible to induce adventitious buds by culturing immature leaves isolated from lateral buds (unpublished data).

Presently, immature leaves inside of winter buds or in lateral buds are the most suitable materials for use for plant regeneration through tissue culture of mulberry for 2 reasons. First, stable regeneration of plantlets was observed only when 2 materials, i.e. immature leaves and leaves of cultured shoots were used. Adventitious bud formation on cultured immature leaves[5,7,12-15,17-19] and leaves of cultured shoots[11] of mulberry was reported for many cultivars by a large number of researchers. Second, a large number of materials can be collected easily from the field. Preparation of the leaves of cultured shoots requires previous shoot culture, which is laborious and takes time as in the case of the culture of leaves from cultured shoots. In the case of immature leaves inside of terminal buds, the number of explants is limited. Only several dozens of terminal buds can be obtained from a mulberry tree grown in the field, whereas several hundreds of winter or lateral buds can be obtained from the same tree[17].

This method can be applied to a limited number of mulberry cultivars (unpublished data). Although there are several reports on the improvement of this culture system[5,7,18], optimum culture conditions, especially medium conditions, have not been fully elucidated. In this report, we studied the optimum medium conditions for adventitious bud formation using immature leaves isolated from winter buds. Furthermore, we examined the suppressive effect of antibiotics and of a herbicide on adventitious bud formation to identify suitable one(s) for selection of transformed plants.

Materials and methods

1) Plant materials

Winter buds of mulberry (*Morus* spp. cv. Shinichinose, Hayatesakari, Kokuso 21, Unryu, Kyukyokusou, Kibajumonji, Garyu, Shidareguwa, Jikunashi, Ryomensou, Shinjiro, Keikansou and Turugisansou) grown in the field were used in this study.

2) Medium

(1) Effect of nitrogen sources[18]

MS medium[8] containing 3% fructose, 5 μM 6-benzylaminopurine (BAP) and 1% agar, and B5 medium[3] containing 2% fructose, and 5 μM BAP and 1% agar were used as basal media. The media were modified by the addition of various concentrations of nitrate ion and ammonium ion (Table 1). The media were adjusted to pH 5.8 and then autoclaved for 15 min before use.

(2) Effect of sugar(s)

MS medium containing 10 μM BAP and 1% agar was used as basal medium. Eight kinds of sugars were tested at various concentrations alone or in combination (Tables 2, 3). The sugar(s) were added into the basal medium before or after 10 min of autoclaving (Tables 2, 3). In the latter case, a concentrated solution of sugar was added into the autoclaved medium. The medium was adjusted to pH 6.0 before autoclaving.

(3) Effect of pH

Liquid MS medium containing 10 μM BAP and 3% fructose was used as basal medium with a filter paper as a supporting material. Various volumes of 1N HCl or 1N NaOH were added into

Table 1. Effect of concentration of nitrate ion and ammonium ion on adventitious bud formation in mulberry

	Basal medium	Concentration of nitrate ion (mM)	Concentration of ammonium ion (mM)	Ratio of nitrate ion concentration to ammonium ion concentration	Adventitious bud formation (%)
Series 1					
	MS	40	0	1:0	0
	MS	40	1	40:1	0
	MS	40	5	8:1	13
	MS	40	10	4:1	33
	MS	40	20	2:1	80
	MS	40	40	1:1	52
Series 2					
	MS	0	20	0:1	–[a]
	MS	10	20	0.5:1	0
	MS	25	20	1.25:1	74
	MS	40	20	2:1	63
	MS	60	20	3:1	50
Series 3					
	B5	25	0	1:0	2
	B5	25	2	12.5:1	4
	B5	25	5	5:1	47
	B5	25	10	2.5:1	57
	B5	25	20	1.25:1	59
	B5	25	40	0.63:1	6
Series 4					
	MS	24	36	0.67:1	34
	MS	30	30	1:1	56
	MS	36	24	1.5:1	54
	MS	42	18	2.33:1	61
	MS	48	12	4:1	34
	MS	54	6	9:1	27
	MS	60	0	1:0	0

Concentrations of nitrate ion and ammonium ion in original MS medium[8] were 39.4 and 20.6 mM, respectively, and those in original B5 medium[3] were 24.7 and 2 mM, respectively.

a): All the leaves plated on the medium died.

the medium before autoclaving. The medium was autoclaved for 10 min followed by the measurement of the pH.

(4) Effect of cytokinins

MS medium containing 1% sucrose, 1% glucose, 1% fructose and 1% agar was used as basal medium. BAP, thidiazuron and kinetin were added before autoclaving. In the cases of 2-isopentenyladenine (2-IP) and zeatin, a concentrated solution was added into the medium after autoclaving.

(5) Effect of other plant hormones

MS medium containing 1% sucrose, 1% glucose, 1% fructose, 1 μM thidiazuron and 1% agar was used as basal medium. 2, 4-dichlorophenoxyacetic acid (2, 4-D) and naphthylacetic acid (NAA) were added before autoclaving. In the cases of other plant hormones, i.e. 3-indoleacetic acid (IAA), indolebutyric acid (IBA), dicamba, gibberellin A_3, abscisic acid, brassinosteroid and methyl jasmonate, concentrated solutions were added into the autoclaved medium.

(6) Effects of antibiotics and a herbicide[19]

MS medium containing 1% sucrose, 1% glucose, 1% fructose, 1 μM thidiazuron and 1% agar was used as basal medium. Antibiotics i.e. kanamycin, geneticin and hygromycin, and a herbicide, i.e. bialaphos, were likewise sterilized by filtering and added into the medium after autoclaving.

3) Leaf culture

One-year-old elongated mulberry branches were collected in winter and stored in a refrigerator at 2.5°C. The winter buds excised from the branches were sterilized with 70% ethanol for 30 s followed by 0.5% sodium hypochlorite solution containing 0.005% Triton X-100 for 15 min. Then, the sterilized materials were rinsed 4 times with sterilized distilled water. Immature leaves were aseptically isolated from winter buds and were placed on the medium. Culture was conducted at 27°C under 14 h light and 10 h dark conditions for 30 days. Frequency of adventitious bud formation (FABF) was calculated as the percentage of leaves on which adventitious bud(s) were formed to the number of leaves that survived after culture.

Results

1) Effects of nitrogen sources[18]

FABF was remarkably influenced by the changes in the concentration of nitrate ion and ammonium ion in the medium (Table 1). Higher FABF than 50% was observed in the medium containing 40 mM nitrate ion and 20 and 40 mM ammonium ion in series 1, in the medium containing 25, 40 and 60 mM nitrate ion and 20 mM ammonium ion in series 2, and in the medium containing 25 mM nitrate ion and 10, 20 mM ammonium ion in series 3 (Table 1). When the concentration of total inorganic nitrogen was adjusted to 60 mM (series 4), higher FABF than 50% was observed in the medium containing 30–42 mM nitrate ion (30–18 mM ammonium ion). These results indicated that the ratio of the concentration of nitrate ion to that of ammonium ion was more important rather than the concentration of either ion. It was reported that the optimum ratio for adventitious

Table 2. Effect of sugar kind on adventitious bud formation of mulberry

Sugar	Adventitious bud formation (%)					
	Shin-ichinose		Hayatesakari		Kokusou 21	
	A[a)]	F[b)]	A[a)]	F[b)]	A[a)]	F[b)]
Sucrose	64	52	28	31	53	54
Glucose	36	55	43	38	55	82
Fructose	63	33	30	40	70	76
Maltose	9	4	6	6	0	7
Lactose	3	10	4	9	4	7
Galactose	0	0	4	14	5	3
Xylose	0	19	1	19	15	24
Mannose	0	0	8	4	0	3
Sorbitol	3	3	0	0	3	6
Mannitol	0	0	0	0	0	0

a): Sugar was added into the medium before autoclaving.
b): Concentrated sugar solution which had been sterilized by filter was added into the medium after autoclaving.

bud formation of apple was in the ranges of 10:1 and 4:1[2]. The most suitable ratio of the concentration of nitrate ion to that of ammonium ion was in the ranges of 1:1 and 3:1. As for the nitrogen source composition, original MS medium is considered to be suitable for adventitious bud formation of mulberry.

2) Effect of sugar(s)

Sucrose, glucose and fructose were more suitable for adventitious bud formation than other sugars (Table 2). The average length of the leaves cultured on the medium containing each one of these 3 kinds of sugars exceeded 18 mm (Table 3). No adventitious bud was observed in the leaves cultured on the medium containing mannitol (Table 2). The average length of the leaves cultured on the medium containing mannitol was shortest (Table 3). It appeared that there was a positive correlation between FABF and the average length of leaves. Meanwhile, there were no apparent differences between the average length of leaves which formed adventitious buds and the length of leaves which did not form them among the leaves cultured under the same sugar conditions (data not shown). These results indicate that the sugars suitable for the growth of cultured leaf were also suitable for adventitious bud formation and that a higher frequency of adventitious bud formation was not caused by more active growth of cultured leaves. The concentrations of sucrose, glucose, fructose and mixtures for suitable adventitious bud formation were in the ranges of 2 and 4% (Table 4).

3) Effect of pH

FABF did not vary appreciably among the media, except for the medium with the highest pH (7.18) in which FABF was 0% (Table 5). It seemed that adventitious bud formation of mulberry was not appreciably affected by the pH of the medium. Since a large amount of precipitation was observed in the highest-pH medium, the inhibition of adventitious bud formation at highest pH was probably caused by nutrient deficiency.

Table 3. Effect of sugar kind on the growth of cultured mulberry (cv. Hayatesakari) leaves

Sugar	Average length of cultured leaves (mm)	
	A[a)]	F[b)]
Sucrose	18.5	21.0
Glucose	21.7	22.8
Fructose	22.7	24.7
Maltose	5.1	6.3
Lactose	7.1	7.8
Galactose	11.4	12.1
Xylose	6.5	10.7
Mannose	6.7	7.3
Sorbitol	3.2	3.6
Mannitol	2.8	2.7

a), b): See Table 2.

Table 5. Effect of pH on adventitious bud formation in mulberry (cv. Kokusou 21)

pH of medium	Adventitious bud formation (%)
3.25	17
3.6	20
3.88	40
4.1	33
4.34	37
4.54	33
5.09	20
5.57	33
6.16	20
7.18	0

Table 4. Effect of concentration of sugar on adventitious bud formation in mulberry (cv. Hayatesakari)

Concentration (%, w/v)	Adventitious bud formation (%)			
	Sucrose	Glucose	Fructose	Mixture[a)]
1	15	17	28	22
1.5	38	38	23	39
2	38	49	44	64
2.5	61	65	42	47
3	67	73	49	67
4	43	56	50	84
5	44	60	33	40

a): Sucrose, glucose and fructose were mixed at the same concentrations.

4) Effect of cytokinins

So far, only BAP had been used as a cytokinin for the induction of adventitious buds from immature leaves of mulberry[5,7,11-13,17-19]. All the 3 adenine-type cytokinins used in this study were less suitable for mulberry adventitious bud formation compared with BAP (Table 6). Thidiazuron, which is a phenylurea compound with a strong cytokinin-like activity[4,9] and which induced adventitious buds on the immature leaves isolated from apical buds of mulberry[15], induced adventitious buds on the cultured immature leaves isolated from winter buds (Table 7). The optimum concentration of thidiazuron which was about 1 μM was approximately 10 times lower than that of BA (Table 7). FABF was higher or similar when 1 μM thidiazuron was used compared with 10 μM BA in 8 of the 10 cultivars examined, although the reverse was observed in 2 cultivars (Table 8).

Table 6. Effect of cytokinins on adventitious bud formation in mulberry (cv. Hayatesakari)

Cytokinin	Concentration (μM)	Adventitious bud formation (%)
BAP	1	0
	5	21
	20	45
2IP	1	0
	5	0
	20	0
Zeatin	1	0
	5	5
	20	0
Kinetin	1	0
	5	0
	20	0

Table 7. Effect of benzyladenine and thidiazuron on adventitious bud formation in mulberry (cv. Hayatesakari)

Concentration (μM)	Adventitious bud formation (%)	
	BAP	Thidiazuron
0.0	0	0
0.05	nt*	0
0.1	nt	0
0.2	nt	13
0.5	nt	25
1.0	0	70
2.0	27	50
5.0	22	0
10	50	nt
20	43	nt

* not tested.

Table 8. Effect of benzyladenine and thidiazuron on adventitious bud formation in mulberry cultivars

Cultivar	Adventitious bud formation (%)	
	10 μM BAP	1 μM Thidiazuron
Unryu	36	34
Kyukyokusou	56	79
Kibajumonji	47	69
Garyu	53	68
Shidareguwa	9	37
Jikunashi	44	16
Ryomensou	52	24
Shinjiro	7	5
Keikansou	27	33
Turugisansou	0	11

5) Effect of auxin, gibberellin A_3, abscisic acid, brassinosteroid and methyl jasmonate

The effect of auxin on FABF was not beneficial (Table 9). High concentrations of auxin suppressed adventitious bud formation and induced callus, except for IAA (Tables 9, 10). Addition of 1 μM abscisic acid promoted adventitious bud formation (Table 11). FABF was not significantly affected by gibberellin A3, brassinosteroid and methyl jasmonate (Tables 12, 13).

6) Effect of antibiotics and a herbicide[19]

All the antibiotics and the herbicide used in this study suppressed adventitious bud formation (Table 14). It was observed that precultured explants were more highly resistant to the antibiotics and the herbicide than the non-precultured ones. However, total suppression of adventitious bud formation was observed in the medium containing the highest concentration of geneticin, hygromycin and bialaphos even in the case of precultured conditions.

Discussion

In a previous paper[6], successful genetic transformation of mulberry by *Agrobacterium* Ti-plasmid was reported, but the frequency was low. One possible modification for improving the mulberry transformation system is to increase the selection efficiency of the transformed organs. In this report, investigations were carried out to determine whether the genes that inactivate geneticin, hygromycin and bialaphos could be used as selection marker genes. Another possible modification is to improve the tissue culture system. We

Table 9. Effect of auxin on adventitious bud formation in mulberry (cv. Hayatesakari)

Concentration of auxin (μM)	Adventitious bud formation (%)				
	IAA	IBA	NAA	2,4-D	Dicamba
0 (Control)	46	32	31	35	46
0.01	58	21	38	28	35
0.1	28	18	38	10	40
1	48	25	18	2.5	7.5
10	48	30	0	2.5	0
100	54	4.2	nt*	nt	nt

*not tested.

Table 10. Effect of auxin on callus formation of mulberry (cv. Hayatesakari)

Concentration of auxin (μM)	Frequency of callus formation (%)				
	IAA	IBA	NAA	2,4-D	Dicamba
0 (Control)	0	0	0	0	0
0.01	0	0	0	0	0
0.1	0	0	0	0	0
1	0	8	28	35	68
10	0	15	93	90	70
100	2	67	nt*	nt	nt

*not tested.

Table 11. Effect of abscisic acid on adventitious bud formation in mulberry (cv. Hayatesakari)

Concentration (μM)	Adventitious bud formation (%)
0	24
0.05	33
0.2	35
1	53
5	35
20	20

Table 12. Effect of Gibberellin A_3 on adventitious bud formation in mulberry (cv. Hayatesakari)

Concentration (μM)	Adventitious bud formation (%)
0	58
0.002	58
0.01	43
0.05	53
0.2	58
1	63
5	45
20	10

Table 13. Effect of brassinosteroid and methyl jasmonate on adventitious bud formation in mulberry (cv. Hayatesakari)

Concentration (μM)	Adventitious bud formation (%)	
	Brassinosteroid	Methyl jasmonate
0	52	51
0.00001	43	nt*
0.0001	47	nt
0.001	63	68
0.01	51	57
0.1	28	53
1	15	63
10	nt	54

*not tested.

showed that the frequency of adventitious bud formation varied with the medium conditions. It may be important to analyze the physiological and molecular biological events that occur during the process of adventitious bud formation.

Callus was induced most efficiently on the medium that did not contain ammonium ion[10], on which no adventitious buds were formed. It is interesting to note that the optimum ratio of nitrate ion to ammonium ion for callus induction of mulberry was different from that of adventitious bud formation, which suggested that metabolic alterations affect directly or indirectly adventitious bud formation.

The kind of sugar rather than the concentration affected FABF. High FABF was observed in the medium containing sugar(s) that promoted vigorous growth of leaves, which indicates that metabolic pathways play a role in adventitious bud formation.

Abscisic acid exerted a beneficial effect on adventitious bud formation. It is generally recognized that abscisic acid promotes the expression of genes related to the resistance to various

Table 14. Effect of antibiotics and a herbicide on adventitious bud formation in mulberry (cv. Hayatesakari)

Concentration (mg/L)	Adventitious bud formation (%)			
	Kanamycin	Geneticin	Hygromycin	Bialaphos
(Not precultured)				
0	45	45	45	45
0.1	nt*	33	nt	38
0.2	nt	42	67	48
0.5	nt	25	21	32
1	nt	4	21	0
2	33	0	0	–
5	46	0	0	–
10	17	0	0	–
20	0	nt	–	–
50	0	nt	–	nt
100	0	nt	nt	nt
(Precultured for 17 days)				
0	43	43	43	43
0.2	nt	50	44	nt
0.5	nt	38	34	31
1	nt	29	28	44
2	53	13	24	47
5	44	10	17	38
10	44	0	0	18
20	38	nt	0	7
50	25	nt	nt	0
100	8	nt	nt	nt

* not tested.
–: All the leaves plated on the medium died.

stresses[1]. Since the culture environment is a kind of stress, the beneficial effect of abscisic acid on adventitious bud formation may be related to stress resistance.

As mentioned above, various physiological reactions controlled by many genes may be related to adventitious bud formation in mulberry. Many genes expressed during the regeneration process have been isolated[20]. This information is important to improve the tissue culture system of mulberry. On the other hand, it was reported that no adventitious buds were formed when immature leaves of sprouting winter buds of mulberry were cultured[13]. We also previously reported that the regeneration potential of immature mulberry leaf was lowered and was completely lost with growth, even if the leaf size was 10 times smaller than that of the mature one[17]. These observations indicate that the expression of genes related to regeneration varies with the development of the organ. On the other hand, adventitious buds were formed whereas callus was scarcely formed on the medium containing 10–100 μM IAA and 1 μM thidiazuron in this study, although callus was induced from cotyledon by 2–10 μM IAA and 2 μM thidiazuron in a previous study[16]. These findings imply that the medium conditions and the endogenous differentiation potential of explant play a role in adventitious bud formation. Since immature leaf is one of the few organs with a potential for differentiation in mulberry, immature leaf culture system of mulberry should be used to analyze processes of regeneration.

References

1) Chandler, P. M. & Robertson, M. (1994): Gene expression regulated by abscisic acid and its relation to stress tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **45**, 113–141.
2) Fasolo, G. H., Zimmerman, R. H. & Fordham, I. (1989): Adventitious shoot formation on excised leaves of in vitro grown shoots of apple cultivars. *Plant Cell Tissue Organ Cult.*, **16**, 75–87.
3) Gamborg, O. L., Miller, R. A. & Ojima, K. (1968): Nutrient requirement of suspension cultures of soybean root cells. *Exp. Cell. Res.*, **50**, 151–158.
4) Huetteman, C. A. & Preece, J. E. (1993): TDZ: a potent cytokinin for woody plant tissue culture. *Plant*

Cell Tissue Organ Cult., **33**, 105–119.
5) Katagiri, K. & Thinh, N. T. (1994): Effect of rotary shaking on the induction of adventitious buds in mulberry leaves cultured *in vitro*. *J. Seric. Sci. Jpn.*, **63**, 278–283.
6) Machii, H. (1990): Leaf disc transformation of mulberry plant (*Morus alba* L.) by *Agrobacterium* Ti plasmid. *J. Seric. Sci. Jpn.*, **59**, 105–110.
7) Machii, H. (1992): Organogenesis from immature leaf cultures in mulberry, *Morus alba* L. *J. Seric. Sci. Jpn.*, **61**, 512–519.
8) Murashige, T. & Skoog, F. (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, **15**, 473–497.
9) Murthy, B. N. S., Murch, S. J. & Saxena, P.K. (1998): Thidiazuron; a potent regulator of in vitro plant regeneration. *In Vitro Cell. Dev. Biol. –Plant*, **34**, 267–275.
10) Oka, S. & Ohyama, K. (1973): Induction of callus and effect of constituents of media on callus formation in mulberry tree. *J. Seric. Sci. Jpn.*, **42**, 317–324 [In Japanese].
11) Oka, S. & Ohyama, K. (1981): In vitro induction of adventitious buds and its modification by high concentration of benzyladenine in leaf tissue of mulberry. *Can. J. Bot.*, **59**, 68–74.
12) Saito, H. (1993): Relationships between leaf age and adventitious bud formation in immature leaf of mulberry. *J. Seric. Sci. Jpn.*, **62**, 435–436 [In Japanese].
13) Saito, H. & Katagiri, K. (1989): Adventitious-bud induction in leaves isolated from winter buds of mulberry. *J. Seric. Sci. Jpn.*, **58**, 46–51 [In Japanese].
14) Sawaguchi, T. et al. (1996): Effect of culture conditions on adventitious-bud formation from cotyledons and primary leaves of mulberry. *J. Seric. Sci. Jpn.*, **66**, 360–363.
15) Thinh, N. T. & Katagiri, K. (1994): Induction of adventitious buds in mulberry leaves by Thidiazuron. *J. Seric. Sci. Jpn.*, **63**, 514–516.
16) Tohjima, F. et al. (1996): Effect of plant hormones on the callus induction from mulberry cotyledons. *J. Seric. Sci. Jpn.*, **65**, 510–513.
17) Yamanouchi, H., Koyama, A. & Machii, H. (1999): Differentiation of adventitious buds on immature leaves isolated from shoots tops of mulberry. *J. Seric. Sci. Jpn.*, **68**, 77–80.
18) Yamanouchi, H., Machii, H. & Katagiri, K. (1993): Effect of inorganic nitrogen sources on adventitious bud formation from leaf cultures in mulberry. *J. Seric. Sci. Jpn.*, **62**, 145–151 [In Japanese].
19) Yamanouchi, H. et al. (1993): Effect of three antibiotics and a herbicide on adventitious-bud formation in immature leaf culture and proliferation of multiple-bud body of mulberry. *J. Seric. Sci. Jpn.*, **66**, 493–496.
20) Zimmerman, J. L. (1993): Somatic embryogenesis; a model for early development in higher plants. *Plant Cell*, **5**, 1411–1423.

(Received for publication, February 10, 1999)