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Efficient Induction of Shoot Organogenesis From Leaves of Mulberry Seedlings Using 2, 3, 5-Triiodobenzoic Acid

Yukio SUGIMURA, Tomoki ADACHI, Eiji KOTANI and Toshiharu FURUSAWA

Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo, Kyoto 606-0962, Japan

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Abstract

Shoot organogenesis from mulberry leaves was examined by using a two-step culture system consisting of seedling culture for obtaining leaf explants and leaf culture for inducing shoot organogenesis. Successful induction of shoots was achieved with an almost perfect induction rate, only when both cultures were carried out in the medium supplemented with 2, 3, 5-triiodobenzoic acid (TIBA) and cytokinin such as 6-benzylaminopurine (BA) and thidiazuron (TDZ). The site of shoot formation was confined to the basal part of a leaf, particularly the surface of midrib near the cut-end of the petiole. Complete whole plantlets were produced from the regenerated shoots. The stimulative effect of TIBA, inhibitor of auxin transport, on shoot organogenesis is discussed in relation to the level of endogenous auxin contained in the used leaf explants.

1. Introduction

Mulberry (*Morus* spp.), a woody feed crop for the silkworm (*Bombyx mori*), is one of the recalcitrant species in terms of tissue culture, although many attempts have been made using explants prepared from leaf, meristem, cotyledon and hypocotyl (Oka and Ohyama, 1981; Kim *et al.*, 1985; Mhatre *et al.*, 1985; Ohyama and Oka, 1987; Saito and Katagiri, 1989; Jain and Datta, 1992). Of various explants, immature leaves in winter buds were a promising explant source for reproducible induction of shoot organogenesis (Saito, 1992; Machii 1992; Katagiri and Thinh, 1994). However, for collection of the immature leaves, mulberry branches with buds must be harvested from a field during winter, and stored in a cold room until use. Dependence on season and the storage of bulky and heavy branches are great disadvantages in an explant source.

As an alternative source of explants, leaves from seedlings is preferable to immature leaves in winter buds, because of no seasonal limitation and easy handling. A previous report demonstrated the induction of adventitious buds using the leaf explants from seedlings grown on the medium containing 6-benzylaminopurine (BA), but there was room for improvement in the frequency of bud formation (Oka and Ohyama, 1981).

In this paper, we describe the shoot organogenesis from leaves derived from mulberry seedlings, which is accomplished by a two-step procedure consisting of seedling culture for obtaining the explant source and leaf culture for inducing adventitious shoot buds directed towards plantlet regeneration. In addition, special attention was paid to the stimulative effect of 2,3,5-triiodobenzoic acid (TIBA), inhibitor of auxin transport, on shoot bud formation from the leaf explants.

2. Materials and methods

Seeds of mulberry (*Morus alba* L. cv. Shin-ichinose) were sterilized with 1% sodium hypochloride solution for 20 min, rinsed with sterile water several times, and sown on the solid LS medium (Linsmaier and Skoog, 1965) supplemented with 6-benzylaminopurine (BA) and/or 2,3,5-triiodobenzoic acid (TIBA). For germination and subsequent seedling growth, they were incubated at 27°C under continuous

fluorescent lighting (3.3 μM m^{-2} s^{-1}). The fully-expanded 1st and 2nd leaves were excised 30 days after incubation and used as explants. These leaves were then cultured on LS medium supplemented with various combinations of growth regulators as follows : 2,4-dichlorophenoxyacetic acid (2,4-D), BA, thidiazuron (TDZ), N^6-(2-isopentenyl)adenine (2ip), zeatin, kinetin, TIBA, and 4-chlorophenoxyisobutyric acid (PCIB). All cultures were maintained at 27°C in the dark. More than 20 explants were cultured in each experiment. For rooting, the regenerated

shoots were transferred to the LS medium without growth regulators and maintained under the same lighting condition as that of the seedling cultures.

3. Results

For induction of adventitious shoot buds from leaf explants, it was important to select leaves from seedlings grown on the medium supplemented with BA (Oka and Ohyama, 1981). On the basis of this result, the induction of shoot organogenesis from seedlings' leaves is thought to be affected by both the conditions of the seedling and the leaf cultures. Therefore, experiments consisting of a two-step culture were set up: seedling culture for the choice of leaf explants, followed by leaf culture for induction of shoot organogenesis.

3.1 Effect of BA and TIBA on the morphology of leaves used as explants

The seedlings were grown on the media supplemented with BA and/or TIBA for obtaining leaf explants with organogenic potency (the 1st step). Seed germination was not inhibited by any of the doses of growth regulators applied in this study. The growth responses after germination were evaluated 30 days after sowing. All seedlings tested were stunted, and the development of roots and leaves was markedly retarded according to the concentration of BA added to the seedling medium. The seedlings grown on the medium with 10 μM BA were abnormal in every organ (**Fig. 1**): small true-leaves with slight serrations and thickened midribs; shortened hypocotyl and epicotyl in its length; undeveloped roots. These morphological alterations by BA were observed in shoots subcultured *in vitro* (Oka and Ohyama, 1981). The combination of BA and TIBA gave similar abnormalities to those induced by a 10 μM BA supplement, except that the 1st and 2nd leaves were somewhat larger. The application of TDZ to the medium was completely harmful for seedling growth, thus being undesirable for the preparation of leaf explants. For the leaf culture (the 2nd step), the 1st and 2nd leaves were excised from the seedlings treated with BA or BA+TIBA and used as explants.

3.2 Organogenesis from leaves excised from seedlings treated with BA

Leaves were excised from seedlings grown on media with 1-10 μM BA, and then cultured on various media containing 2,4-D and/or BA. Different kinds of proliferative responses were observed 1 month after culture (**Table 1**). Using leaves from seedlings treated with and without BA, calli and/or adventitious roots were induced on the media with 2,4-D + BA and 2,4-D alone. Shoot induction without calli was observed only when leaves from seedlings treated with BA were cultured on the medium with BA alone, which was clearly the BA-dependent response. The effectiveness of BA was completely nullified by the addition of 2,4-D. Calli were initiated from the surface of leaf lamina and midrib, whereas adventitious roots were induced from the surface of midrib and the basal cut end of the midribs (**Fig. 2**). Profuse adventitious roots were initiated from a mass of callus. The site of shoot formation was confined to the basal part of a leaf, particularly the surface of the midrib near the cut end. Shoot primordia were never produced at other parts of a leaf in the present conditions.

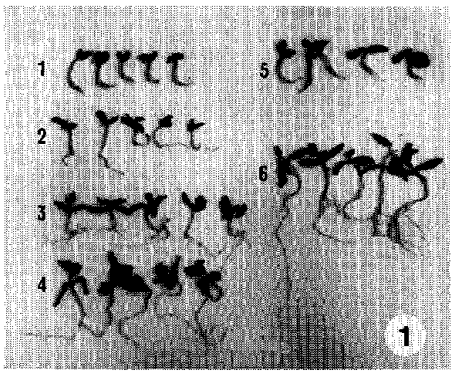

Fig. 1 Effect of BA and TIBA on the growth of mulberry seedlings.
Seedlings were grown on various media supplemented with (1) 10 μM BA, (2) 1 μM BA, (3) 0.1 μM BA, (4) 1 μM TIBA, (5) 10 μM BA and 1 μM TIBA. (6) control.

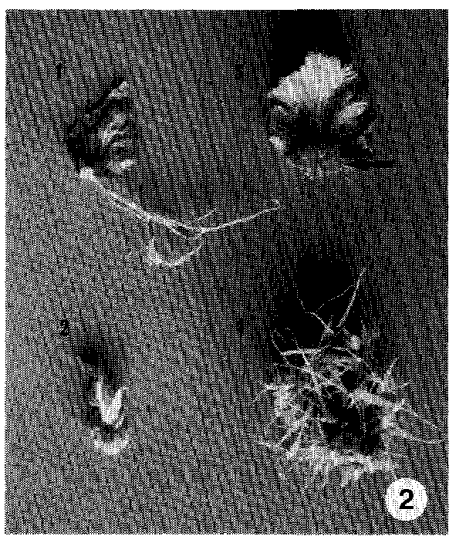

Fig. 2 Various proliferative responses induced from leaves of mulberry seedlings.
(1) adventitious roots, (2) callus, (3) adventitious shoots (arrow), (4) callus mass with adventitious roots.

It is evident that the addition of BA to the media for seedling and leaf cultures is indispensable for successful shoot organogenesis. Instead of BA, other types

Table 1 Various proliferative responses to 2,4-D and BA supplemented to the media for the seedling and the leaf cultures.

Seedling culture	Leaf culture		Induction rate (%)				
BA (μM)	2,4-D (μM)	BA (μM)	Callus	Root	Callus with roots	Shoot	No response
0	1	1	14	0	86	0	0
	10	0.1	0	14	86	0	0
	1	0	0	17	83	0	0
	10	0	0	0	100	0	0
	0	1	0	0	0	0	100
	0	10	0	0	0	0	100
	0	0	0	0	0	0	100
1	1	1	29	0	71	0	0
	10	0.1	0	0	100	0	0
	1	0	0	14	86	0	0
	10	0	0	0	100	0	0
	0	1	0	17	0	17	66
	0	10	0	0	0	17	83
	0	0	0	14	0	0	86
10	1	1	0	0	100	0	0
	10	0.1	0	0	100	0	0
	1	0	13	37	50	0	0
	10	0	0	0	100	0	0
	0	1	0	0	0	17	83
	0	10	0	0	0	29	71
	0	0	0	14	0	0	86

Table 2 Shoot induction by various cytokinins.

	Cytokinin added		Shoot Induction (%)
Exp. 1	1μM	BA	15
	1μM	Zeatin	5
	1μM	Kinetin	0
	10μM	2ip	0
Exp. 2	1μM	TDZ	13
	10μM	TDZ	38
	10μM	BA	13

Leaves from seedlings treated with 10μM BA were used as explants.

Table 3 Effect of anti-auxins on shoot induction.

Cytokinin		Anti-auxin		Shoot Induction (%)
10μM	BA			18
10μM	BA	0.1μM	TIBA	26
10μM	BA	1μM	PCIB	13
10μM	BA	0.1μM	PCIB	6
10μM	TDZ			38
10μM	TDZ	0.1μM	TIBA	50
10μM	TDZ	1μM	PCIB	13
10μM	TDZ	0.1μM	PCIB	30
		10μM	TIBA	0
		1μM	TIBA	0
		0.1μM	TIBA	0
		1μM	PCIB	0
		0.1μM	PCIB	0

Leaves from seedlings treated with 10μM BA were used as explants.

of cytokinin were also tested in leaf culture. A better result was obtained by the supplement of TDZ at 10 μM concentration (**Table 2**), but no or little organogenesis occurred when zeatin, 2ip and kinetin were substituted for BA.

3.3 Stimulative effect of anti-auxins on shoot induction

For the blockage of endogenous auxin activity, anti-auxins (TIBA and PCIB) were tested by two sets of experiments as follows:

Experiment 1: Leaves grown on the medium with BA alone were cultured on the media with the combination of anti-auxin and cytokinin (BA and TDZ). Compared with a single application of BA or TDZ, the shoot formation was markedly stimulated by the addition of TIBA, the combination of TDZ and TIBA in particular (**Table 3**). Such stimulative effect was not found in PCIB.

Experiment 2: Leaves grown on the medium containing BA and TIBA were chosen as explants, and then cultured on the media with both of TDZ and

Table 4 Shoot induction stimulated by TIBA added to the media used for the seedling and the leaf cultures.

Seedling culture	leaf culture	Shoot Induction (%)
10μM BA	10μM TDZ	40
	10μM TDZ+0.1μM TIBA	66
10μM BA+1μM TIBA	10μM TDZ	60
	10μM TDZ+0.1μM TIBA	93
1μM TIBA	10μM TDZ	7
	10μM TDZ+0.1μM TIBA	13
None	10μM TDZ	0
	10μM TDZ+0.1μM TIBA	0

TIBA. The percentage of shoot bud induction was remarkably increased up to more than 90% (**Table 4**). This stimulative effect of TIBA was confirmed in repeated experiments.

3.4 Whole plantlets from regenerated shoots

The regenerated shoots with a few juvenile leaves were excised from the initial explants and transferred to the medium without plant growth regulators. Roots were initiated from the basal part of the shoots. Eventually, complete whole plants with normal morphology in the shoots and leaves were produced, indicating that plant growth regulators (BA, TDZ and TIBA) used for shoot organogenesis were not necessary for root formation from the regenerated shoots.

4. Discussion

Using leaves from mulberry seedlings as an explant source, it was clearly demonstrated that a high percent of shoot induction was achieved in the specified conditions. Shoots were regenerated only when leaves derived from seedlings treated with BA were cultured on the medium with BA or TDZ (**Tables 1 and 2**). Apparently, the level of BA in the seedling culture medium had a definitive effect on shoot bud initiation. Some physiologically favourable status for bud initiation may be established in the seedlings by BA treatment. For successful formation of shoots, it was crucial that the explants were subject to culture on the medium with BA or TDZ. These indicate that the continuous supply of highly active cytokinins such as BA and TDZ is required for the shoot induction throughout the whole culture step.

Both BA and TDZ exogenously applied appear to be effective in counteracting the endogenous auxin levels. Two types of anti-auxins were examined for the blockage of endogenous auxin activity. A drastic increase in shoot formation was observed when TIBA was applied together with BA or TDZ (**Tables 3 and 4**). Considering that TIBA is an inhibitor of auxin transport, the shoot organogenesis is closely associated with the basipetal transport of endogenous auxin.

The formation site of shoot buds was confined to the basal part near cut end, suggesting that the endogenous auxin level is unevenly distributed within the entire leaf explant. Oka and Ohyama (1981) pointed out the existence of some specific potency of producing shoot buds in that part. It is likely that a relatively high level of endogenous auxin is present in the apical part, but that a low level is contained in the basal part. The application of TIBA may block the auxin efflux from the apical to the basal part, so that the balance between the levels of auxin and cytokinin become favourable for the initiation and development of shoot buds at the basal part of a leaf.

In conclusion, the almost perfect induction rate in shoot organogenesis could be achived only when the combination of cytokinin (BA or TDZ) and TIBA was applied to the medium for both seedling and leaf cultures. The culture system, described here, is useful for large scale multiplication, since multiple shoots were directly generated without the callusing passage and somaclonal variation could be minimal. In addition, new possibilities in varietal improvement through genetic manipulation can be opened by the present culture procedure established due to the ease of high frequency of organogenesis.

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