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Induction of Multiple shoots by Shoot Apex Culture in *Magnolia obovata* Thunb.

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Shoot apex of *Magnolia obovata* Thunb. was cultured on the modified Murashige and Skoog's(MS) medium supplemented with the combinations of IBA and BAP or NAA and BAP in various concentrations. Multiple shoots were induced successfully on the half strength MS reducing nitrate to one fourth(1/4 N-1/2 MS) medium containing 4 μM IBA and 5 μM BAP after 40 days of culture. The multiple shoots obtained were divided into small nodal explants and further subcultured on fresh medium. Many axillary buds were induced from the explant after 20 days of culture, and they succeedingly grew actively and developed multiple shoots. Culturing the axillary bud together with the stem tissue was effective for facilitating the induction of multiple shoots.

Introduction

Magnolia obovata Thunb., a broad-leaved tree which belongs to the Family Magnoliaceae, is valuable for garden or park planting in Japan. Its wood is used as a material for furniture and various industrial arts, and the bark contain valuable medicinal compounds[1]. This species has been propagated mostly through seeds so far. However, the germination rate of the seeds is relatively low(about 35%), and the propagation by cuttings is also difficult because of its poor rooting ability[2]. Therefore, propagation of *M. obovata* by using tissue culture techniques is desired.

The culture of shoot apex is useful for the micropropagation of seedlings since many explants are available from a mature tree and sterilization of the explants is also easy. In addition, there is an advantage in the culture of shoot apex in that it contains less polyphenolic compounds often inhibiting explant growth, although some woody plants such as *Fagus* and *Zelkova* usually contain large amounts of them in their shoot[3]. The culture of shoot apex has been carried out in some broad-leaved tree species, such as *Malus pumila* Mill. var. *domestica* C. K. Schn.[4], *Paulownia tomentosa* Steud[5] and *Aesculus carnea* Hayne[6]. In *Magnolia virgininana, M. frasei* and *M. accuminata*, Merkle and Wiecko[7] successfully induced somatic embryos from immature seeds but failed to regenerate plants. In the Magnoliaceae species, there is only one report on successful plant regeneration from somatic embryos of yellow poplar(*Liriodendron tulipifera* L.)[8]. In *Magnolia* spp., however, there are no reports concerning the *in vitro* propagation using shoot apex

from mature trees.

In the present study, various cultural conditions of shoot apex that effectively induce multiple shoots in *M. obovata* were examined.

Materials and Methods

1. *Preparation of explants*

About 5 cm long shoots were collected from a 60-year-old tree of *Magnolia obovata* Thunb. growing in the campus of Utsunomiya University. The shoots were immersed in a solution of neutral detergent for 5 min. After washing with running tap water, they were surface-sterilized with 70% ethanol for 30 sec., with 3% sodium hypochlorite solution containing a few drops of Tween 80 for 20 min., and then rinsed three times with sterilized distilled water. After drying on a sterilized filter paper, stipules were removed and a 4 mm long shoot apex with four leaf primordia was excised.

2. *Screening of basal medium to culture shoot apex*

Four media were used for screening the basal medium; Murashige and Skoog's (MS)[9], Linsmaier and Skoog's (LS)[10], woody plant (WP)[11] and broad-leaved tree (BT)[12]. Additional 3 modified MS media and one modified LS medium were also examined; the concentration of each composition of MS in half (1/2 MS), half nitrate of MS (1/2 N-MS), half strength MS reducing nitrate to one fourth (1/4 N-1/2 MS) and the concentration of each composition of LS in half (1/2 LS). Five μM 6-benzylaminopurine (BAP), sucrose (30 g/l) and agar (7 g/l) were added to all the media, and the pH was adjusted to 5.8 before autoclaving.

3. *Culture of shoot apex*

Shoot apex was cultured on the 1/4 N-1/2 MS basal medium to induce multiple shoots. An auxin of 1-naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA) was added to the basal medium in various concentrations of 0-10 μM together with 4 levels of BAP (0, 1, 5, 10 μM), respectively. The explants were subcultured at intervals of 30 days up to 150 days of culture. Ten explants were used in each treatment. In all experiments, cultures were maintained at 25°C under a 16 hrs-photoperiod (4, 000 lux).

4. *Culture of multiple shoots*

Multiple shoots obtained were divided into small pieces of about 1.0 cm in length with an axillary bud and cultured on the 1/4 N-1/2 MS basal medium containing 4 μM IBA and 5 μM BAP. Some of the multiple shoots were cultured on the same medium without dividing. In order to examine the effect of stem tissues on the induction of multiple shoots, an axillary bud with or without stem tissues (about 5×5×5 mm) was subcultured on the same medium as used for axillary bud culture.

Results and Discussion

1. *Screening of basal medium*

The results of screening basal medium suitable for culturing the shoot apex of *M. obovata* are shown in **Table 1.** Woody plant and BT media were not effective for the flushing of the shoot apex, the flushing rate (FR) being less than 30% after 30 days of culture. Most of the explants cultured on both media formed callus at first, then browned and finally died. Murashige and Skoog's and LS media gave FR of 60% and 50%, respectively, although multiple shoots were not induced. Among the 4 modified media, 1/4 N-1/2 MS medium was the most effective for the flushing of the shoot apex, the FR being 83% (**Table 2**); the medium was chosen for further experiments, though it did not allow shoot elongation.

Table 1. Screening of the basal medium suitable for the culture of shoot apex of *Magnolia obovata.*

Medium	No. of explants	FR(%)	No. of browned or dead explants
MS	30	60	12
LS	30	50	15
WP	30	27	22
BT	30	17	25

FR=flushing rate. Explants were cultured for 30 days on the solid medium supplemented with 5 μM BAP.

Table 2. Effects of the modified MS and LS media on the growth of shoot apex of *Magnolia obovata.*

Medium	No. of explants	FR(%)	No. of browned or dead explants
1/2 N-MS	30	63	8
1/2 MS	30	70	9
1/4 N-1/2 MS	30	83	5
1/2 MS	30	67	10

FR=flushing rate. Explants were cultured for 30 days on the solid medium supplemented with 5 μM BAP.

2. *Culture of shoot apex*

Callus formation from the shoot apex was observed in most of the hormonal combinations of NAA, IBA and BAP(**Table 3**). In particular, NAA caused either browning of the explants or callus formation even at its lower concentrations. On the other hand, 5 μM and 10 μM BAP alone caused bud flushing without shoot elongation. A high concentration(10 μM) of BAP rather caused browning.

When 0.1 μM IBA and 1-5 μM BAP were added to the basal medium, both bud flushing and shoot elongation occurred most frequently. In the medium containing 1 μM IBA, most of the explants did not show any shoot elongation, although bud flushing frequently occurred. At concentrations above 5 μM, IBA induced callus formation or browning of the explants rather than shoot elongation. Similarly, 1 μM BAP frequently caused browning of the explants. Eventually, 4 explants cultured on the medium with 0.1 μM IBA and 5 μM BAP showed active shoot elongation after the bud flushing.

3. *Induction and culture of multiple shoots*

In order to induce multiple shoots effectively, the shoot apices were further cultured at various concentrations of IBA(0.5, 3, 4 μM). The results are shown in **Table 4**. The medium with 0.5 μM IBA and 5 μM BAP gave a better result concerning the shoot elongation. However, these explants formed only a few leaves without axillary bud growth. The formation of axillary buds occurred only in the medium containing 4 μM IBA and 5 μM BAP.

This medium gave the flushing of the shoot apex after 20 days of culture, and the gradual elongation of the shoot after 35 days of culture, subsequently induced axillary bud formation as well as defoliation from the explant a few days after that(**Fig. 1**). Thereafter, the main shoot and axillary buds grew actively and developed into multiple shoots after 40 days of culture(**Fig. 2**).

Furthermore, the effect of the 1/4 N-1/2 MS medium containing 4 μM IBA and 5 μM BAP on the formation of multiple shoots was reexamined. Some explants that failed to form multiple shoots on other media were transferred onto this medium and subcultured. As a result, out of ten explants used, six of them successfully regenerated many axillary buds after 20 days of culture and developed

Table 3. Effects of plant growth regulators on the growth of shoot apex on the 1/4 N-1/2 MS medium.

PGR (μM)			Flushing	Shoot elongation	Callus formation	Browned or dead explants
IBA	NAA	BAP				
0		0	0	0	0	10
0		1	1	1	0	8
0		5	8	0	0	1
0		10	4	0	0	6
0.1		0	1	0	1	7
0.1		1	1	3	0	6
0.1		5	2	4	2	2
0.1		10	0	1	3	6
1		0	2	0	1	7
1		1	2	1	1	6
1		5	6	1	2	1
1		10	0	0	2	8
5		0	0	1	3	7
5		1	1	1	2	6
5		5	3	0	3	4
5		10	0	0	5	5
10		0	0	0	2	8
10		1	0	0	4	6
10		5	1	0	5	4
10		10	0	0	1	9
	0.1	0	0	0	2	8
	0.1	1	1	0	4	5
	0.1	5	1	0	6	3
	0.1	10	0	0	3	7
	1	0	0	0	3	7
	1	1	0	0	3	7
	1	5	1	0	3	6
	1	10	0	0	1	9
	5	0	0	0	1	9
	5	1	0	0	2	8
	5	5	1	0	4	5
	5	10	0	0	1	9
	10	0	0	0	10	0
	10	1	0	0	1	9
	10	5	0	0	7	3
	10	10	0	0	4	6

Shoot apex was cultured for 150 days. Ten explants were used in each theatment.

into multiple shoots through the same process as described above. These results apparently confirmed that the 1/4 N-1/2 MS medium containing 4 μM IBA and 5 μM BAP is effective for the formation of multiple shoots.

4. *Effect of stem tissues on induction of multiple shoots*

Axillary buds with or without stem tissues were transferred to the same medium as used for axillary bud formation. As shown in **Table 5**, the axillary buds without stem tissues did not grow actively, whereas those with stem tissues showed active growth. In addition, many fresh buds were induced from an axillary bud after 20 days of culture, and thereafter they developed into multiple shoots after 30 days in 12 explants out of 15 axillary buds cultured. Furthermore, nodal explants

Table 4. Effects of plant growth regulators on the formation of multiple shoots.

IBA (μM)	BAP (μM)	Flushing	Shoot formation		Callus formation	Browned or dead explants
			single	multiple		
0.5	0	1	0	0	0	9
0.5	1	1	4	0	0	5
0.5	5	2	7	0	0	1
3	0	2	0	0	7	1
3	1	3	2	0	5	0
3	5	4	1	0	4	1
4	0	1	0	0	8	1
4	1	4	1	0	2	3
4	5	5	0	4	0	1

Shoot apex was cultured for 150 days. Ten explants were used in each treatment. Numerals indicate the number of explants. Basal medium: 1/4 N-1/2 NS.

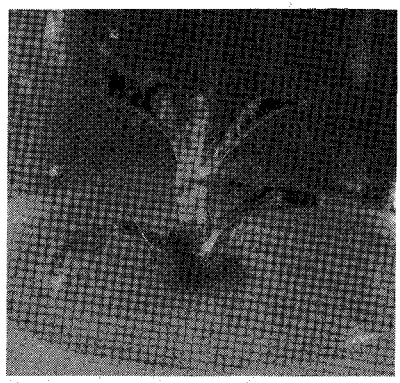

Fig. 1 Formation of axillary buds from shoot apex *M. obovata* cultured for 30 days on the 1/4 N-1/2 MS medium containing 4 μM IBA and 5 μM BAP.

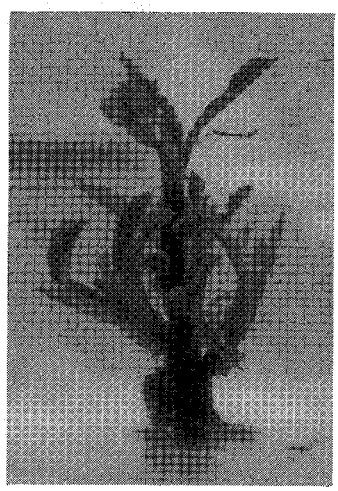

Fig. 2 Multiple shoots formation from shoot apex of *M. obovata* cultured for 40 days on the 1/4 N-1/2 MS medium containing 4 μM IBA and 5 μM BAP.

prepared from the 12 multiple shoots again induced many axillary buds 20 days after culture. Finally, 62 multiple shoots were obtained from nodal explants after 60 days of culture, while only 9 were obtained from the axillary buds without stem tissues. These results indicate that the induction and subsequent growth of the axillary buds were promoted by the tissue continuity between bud and stem tissue.

Table 5. Effects of explants with or without stem on the formation of multiple shoots.

Explants	No. of cultured axillary buds	No. of multiple shoots		
		30	(Days)	60
With stem	15	12		62
Without stem	15	1		9

The axillary buds were cultured on the 1/4 N-1/2 MS medium containing 4 μM IBA and 5 μM BAP and subcultured on the fresh medium at intervals of 30 days throughout the culture period of 60 days.

In the present study, multiple shoots were successfully obtained by the induction of axillary buds from the shoot apex cultured on the 1/4 N-1/2 MS medium. Concerning the formation process of multiple shoots, an interesting phenomenon was observed; when undivided whole multiple shoots were transplanted on the basal medium, shoot tip necrosis occurred several days after the flushing of newly induced axillary buds. Of interest is that the flushed axillary buds showed more active growth after the shoot tip necrosis probably due to the temporal inhibition of apical dominance. From these results, we consider that the excision of the terminal bud of the main shoot immediately after the flushing of axillary buds is an effective method for the formation of multiple shoots from the shoot apex.

Further studies for screening rooting medium of the multiple shoots are needed.

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《和文要約》

ホオノキの茎頂培養による多芽体誘導

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ホオノキの茎頂培養による多芽体誘導条件の検討を行った．成木頂芽から摘出した茎頂を，オーキシン(IBA あるいは NAA)とサイトカイニン(BAP)を組み合わせて添加した改変 MS 培地(窒素濃度を 1/4，他の塩類濃度を 1/2 に改変)で培養した．培養 40 日後，IBA 4 μM と BAP 5 μM を組み合わせた処理区において，多芽体の形成が観察された．誘導された多芽体は，一つずつの腋芽に分割して同組成の培地に移植し，培養した．移植された腋芽は活発に成育し，継代培養 20 日後には多芽体の形成が観察された．この際，腋芽に多芽体の茎組織の一部分を付けて培養することにより，多芽体が効率的に誘導された．