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Hormonal Responses of Petioles and Embryos in *Ginkgo biloba* Cultures

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Introduction

Ginkgo biloba L. is highly important because the medicinal compounds, i.e. ginkgolides A, B, C, J, and bilobalide, are present in the leaves. Numerous publications have appeared on the pharmacology of these compounds. By contrast, little work has been done on *in vitro* cultures of *G. biloba*. Calli have been induced from male and female haploid tissues[1,2], and from zygotic embryos[3,4]. In addition, direct embryogenesis from microspores and female haploid protoplasts have been reported[5,6]. However, there is no published report of *in vitro* plant regeneration from *Ginkgo* explants. The aim of this work is to establish a protocol for the regeneration of plantlets from *G. biloba* explants or cultured cells.

Materials and Methods

1. Plant materials

Young petioles were collected from the end of May until the beginning of June from a *G. biloba* tree in the Univ. of Tokyo. Seeds were collected in December and used immediately or stored at 3°C for 3 months.

2. Culture of petioles and embryos

Petioles were cut into about 1 cm long segments. They were soaked in 70% ethanol for 1 min. and in 1% sodium hypochlorite solution for 15 min. Then, they were washed with sterile distilled water 3 times. Stony layers of the seeds were removed and megagametophytes were surface sterilized as described above, and then embryos were aseptically excised. Linsmaier & Skoog's mineral salts[7] supplemented with 1.0 mg/*l* thiamine chloride, 100 mg/*l* myo-inositol, 30 g/*l* sucrose and 10 g/*l* agar were used as the basic medium (LS1). Plant growth regulators of 2,4-dichlorophenoxyacetic acid (2,4-D), indoleacetic acid (IAA), indolebutyric acid (IBA), naphthaleneacetic acid (NAA), kinetin (K) and 6-benzyladenin (BA) were added in various combinations. The pH of all media was adjusted to 5.8–6.0 before autoclaving. IAA and IBA were filter-sterilized and added to the media before gelling. Both petiole and embryo explants were cultured on 10 ml of medium in 20×150 mm test tubes. Tubes were closed with silicon plugs. All cultures were maintained at 26°C under a 16-h light/8-h dark photoperiod of 1200 lux.

3. Culture of callus

Calli derived from excised embryos on the media 7 and 9 (Table 2) were subcultured every month on the same medium. After 2 to 3 times of subculture, small pieces of the calli were transferred to fresh media indicated in Table 3 and cultured in the same condition in order to obtain organogenesis from calli.

LS1 medium and LS1 medium without ammonium nitrate (LS2) were used as basic

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media. Plant growth regulators of 2,4-D, IAA, IBA, NAA, K, BA, zeatin (Z), gibberellic acid (GA_3) and abscisic acid (ABA) were added in various combinations. The pH of all media was adjusted to 5.8–6.0 before autoclaving. IAA, IBA, Z, GA_3 and ABA were filter-sterilized and added to the media before gelling.

Results

Responses of petioles and excised embryos to plant growth regulators are shown in Table 1 and Table 2, respectively. Organogenesis from petiole explants was not observed on the media used. Green calli were easily obtained from both petiole and embryo explants in most of the hormonal combinations of NAA, 2,4-D, BA and K, and the calli were able to be subcultured on the same media. In addition, these green calli changed to white in color when incubated in the dark. When exposed to light, the white calli gradually became green again. This capacity was persistent over one year. Single addition of IAA, IBA and BA did not promote callus growth in both explants, but promoted germination of excised embryos. First experiment was done using embryos excised from the seeds soon after they were detached from the tree in December. At first a little pale green calli emerged throughout the embryo in almost all the explants. On the medium 2, 3 and 8, the calli grew no more and germination was observed. On the medium 1, only rooting was observed. On the medium 4, 5, 6, 7 and 9, the calli continued growing but germination was not seen. The second experiment was performed in March using embryos excised from the seeds stored at 3°C until then. The embryos were rather bigger in March than in December. The media tested were No. 1, 3 and 6. On the media 3 and 6, growth responses of the embryos were the same as observed in the first experiment in December. However, on the medium 1, embryos showed germination. This result was different from that observed in the first experiment.

Table 1. Hormonal responses of petiole explants

Plant growth regulators (mg/*l*)	Growth responses*
NAA (1.0)	Green callus formation
IAA (1.0)	Dead
IBA (1.0)	Dead
BA (1.0)	Dead
2,4-D (0.5)+BA (0.4)	Green callus formation
NAA (1.0)+BA (0.2)	Green callus formation

* Explants were cultured for 30 days.

Table 2. Hormonal responses of excised embryos

Medium No.	Plant growth regulators (mg/*l*)	Growth responses*
1	None	R, [G′]
2	IAA (1.0)	G
3	IBA (1.0)	G
4	NAA (1.0)	C^+
5	2,4-D (1.0)	C^+
6	2,4-D (0.5)+K (0.4)	C^+
7	NAA (1.0)+BA (0.2)	C^+
8	IBA (1.0)+BA (0.2)	G
9	NAA (2.0)+K (5.0)	C^+

* Embryos were cultured for 40 days.
C^+: green callus formation, G: germination, R: only roots development, [G′]: Germination was observed only when embryos exciesed from the seeds stored at 3°C for 3 months were used.

Table 3 shows the effects of plant growth regulators on growth responses of the embryo-derived calli. When only cytokinins were added, the calli gradually became brown and at last died. Single addition of IAA or IBA, as well as no addition of plant growth regulators showed the same result. On the other hand, the combinations of cytokinins and rather low concentrations of NAA permitted callus growth, but it was slow. In all the media tested, organogenesis from calli was not observed at all.

Discussion

This study shows that induction of *in vitro* organogenesis from *Ginkgo* is difficult. By contrast, dedifferentiation easily occur-

Table 3. Hormonal responses of embryo-derived calli on modified LS media

Basal media	Plant growth regulators (mg/*l*)	Growth responses*	
		Callus growth	Organogenesis
LS1	BA (1.0)	D	—
LS1	K (1.0)	D	—
LS1	Z (1.0)	D	—
LS1	BA (0.5)+Z (0.5)	D	—
LS1	K (0.5)+Z (0.5)	D	—
LS1	BA (0.5)+K (0.5)+Z (0.5)	D	—
LS1	BA (0.5)+Z (0.5)+NAA (0.1)	Slow	—
LS1	K (0.5)+Z (0.5)+NAA (0.1)	Slow	—
LS1	BA (0.5)+K (0.5)+Z (0.5)+NAA (0.1)	Slow	—
LS1	IAA (1.0)	D	—
LS1	None	D	—
LS2	BA (0.4)	D	—
LS2	BA (0.4)+NAA (0.05)	Slow	—
LS2	IBA (1.0)	D	—
LS2	BA (2.0)+NAA (0.2)+ABA (0.1)	Slow	—
LS2	BA (2.0)+NAA (0.2)+GA_3 (0.2)	Slow	—

D: dead, —: not observed.

* Calli were cultured for 30 days, then transferred to the same fresh media and cultured for 30 days again.

red in petiole and embryo explants on the media supplemented with 2,4-D or NAA (Table 1, Table 2).

In the first experiment performed in December, excised embryos showed germination on the media containing IAA or IBA alone, while the embryos did not germinate on hormone-free medium. However, in the second experiment performed in March, the excised embryos, which became bigger than those in the first experiment, germinated also on the hormone-free medium (Table 2). These results suggest that in December, as soon as seeds are detached from the tree, embryos were yet immature and not ready to germinate, but embryos continued growing in the seeds and they were ready to germinate by themselves in March. It was also found that IAA and IBA caused premature germination.

NAA or 2,4-D seemed to be necessary for continuous growth of the calli. Single addition of IAA and IBA did not permit the growth of the calli. It suggests that the calli induced from petioles and embryos of *Ginkgo* require not IAA or IBA, but 2,4-D or NAA as an auxin for their growth.

In the present study, redifferentiation from calli and direct organogenesis from petiole explants were not observed at all. In order to obtain organogenesis from these explants, medium components and culture conditions such as light and temperature, are to be further investigated.

Summary

Petioles and embryos of *Ginkgo biloba* were cultured on modified Linsmaier & Skoog medium supplemented with various kinds and concentrations of auxins and cytokinins. Calli were easily obtained on the medium supplemented with various combinations of 2,4-dichlorophenoxyacetic acid or naphthaleneacetic acid as an auxin, and kinetin or 6-benzyladenine as a cytokinin. The calli grew rapidly and were able to be subcultured on the same medium. These calli were white in color in the dark, but exposed to light, they

easily changed to green. Organogenesis was not observed in petiole explants, but embryos excised from the seeds immediately after their falling to the ground showed germination and leaf expansion on the medium supplemented with indoleacetic acid (IAA) or indolebutyric acid (IBA) alone. When embryos excised from the seeds stored at 3°C for about 3 months were used, they germinated and seedlings were obtained not only on IAA or IBA containing medium but also on a hormone-free one. Redifferentiation from calli was not seen by combining of growth regulators or modifying the mineral salt in the medium.

Key words: *Ginkgo biloba*, phytohormone, dedifferentiation, germination

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イチョウ培養系における葉柄及び胚の植物ホルモン応答

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要　　旨

イチョウの葉柄ならびに胚を外植片として用い，改変 Linsmaier & Skoog 培地にオーキシンとサイトカイニンの種類と濃度を変えて添加した培地で培養した。オーキシンとして 2,4-D または NAA，サイトカイニンとして BA またはカイネチンを組み合わせて添加すると脱分化が容易に起こり成長の速いカルスが得られ，継代して培養が可能であった。このカルスは，暗所では白色をしているが光照射により容易に緑化した。葉柄からの分化は観察されていないが，落下直後の果実から取りだした胚を IAA または IBA のみを加えた培地で培養したところ，発芽して葉の

展開が見られる場合があった。また，3°C で 3 カ月間保存した種子から取りだした胚を用いた場合，ホルモンを加えない培地においても発芽がみられ完全な幼植物体になるものもあった。一方，カルスからの再分化は，成長調節物質の組み合わせや培地の無機塩の改変によっては認められなかった。

キーワード：イチョウ，植物ホルモン，脱分化，発芽

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Hormonal responses of petioles, embryos and green calli induced from these explants in *Ginkgo biloba* cultures were investigated. Addition of 2,4-D or NAA caused green callus formation under illumination from both explants. These calli were able to be subcultured on the same medium. Addition of IAA or IBA alone promoted immature germination of excised embryos. Mature embryos germinated on hormone-free medium. Induced calli did not redifferentiate by combining of growth regulators or modifying the mineral salt in the media.

Measurement of the Properties of Standing Trees with Ultrasonics and Mapping of the Properties

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The MOE (Modulus of Elasticity) of standing trees was measured with ultrasonics. The species for the experiment were Todo-fir (*Abies sachalinesis*) and Larch (*Larix leptolepis*). There were differences between the values of the MOE for certain forest stands at different locations and of different ages. The velocities of ultrasonics could be used instead of the MOE as an index for the properties of standing trees. But more data are needed to clarify the relationships between the diameters at breast height, tree heights and the MOE. Maps of the MOE for standing trees in forest stands could be made by means of the estimated density. These maps could provide very useful information for bucking and timber production.