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Plant Regeneration from Axillary Buds of Plants and Calli of Mature Embryos in *Glehnia littoralis**

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Abstract : *Glehnia littoralis* Fr. Schm. is very popular as a flavored wild vegetable and the roots are a useful crude drug. However, cultivation of *Glehnia* is extremely expensive because of the short harvest period. This work was conducted to clarify the propagability of *Glehnia* from different explants and mature embryos isolated from seeds. Only axillary buds formed about 30 shoots with good root formation in the medium containing BA 1-5 μM+NAA 5 μM. By mature embryo culture, about 40 shoots with 80% root formation per embryo explants were obtained on media containing BA 1 μM+NAA 5 μM. For callus formation from mature embryos, media containing 2, 4-D 1 μM or NAA 5 μM were best, showing that callus was formed from about 90% of embryo explants. *Glehnia* callus produced by embryo explants induced about 45 embryoids in suspension culture for 4 weeks when cultivated on medium containing 2, 4-D, Then, each embryoid formed about 30 shoots with roots in the MS solid medium. These multiple shoots with roots vigorously grew into plants following acclimatization. Thus, induction of multiple shoots derived from embryoids through calli, which were formed from one mature embryo, may be an effective method of mass producing *Glehnia* plants compared to formation of multiple shoots from axillary buds or mature embryos.
Key words : Axillary buds, Embryoids, *Glehnia littoralis*, Mature embryos, Plant regeneration, *Umbelliferae*.

野生ハマボウフウ（*Glehnia littoralis* Fr. Schm.）の腋芽および成熟胚から誘導したカルスによる植物体増殖

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要　旨：ハマボウフウ（*Glehnia littoralis*）は，香りの良い野生野菜として人気があり，生薬としても有用である．しかし，栽培ハマボウフウの収穫期が短期間であるため値段が非常に高い．ここでは，植物の種々の器官と成熟胚からの植物体増殖を試みた．植物の器官では，腋芽のみがBA 1～5 μM＋NAA 5 μMを含むMS固体培地で10週間培養すると1個当たり約30本の不定芽を形成し，それからの不定根も良く形成された．成熟胚の場合は，BA 1 μM＋NAA 5 μMを含むMS固体培地に10週間置床すると成熟胚1個当たり約40本の不定芽が得られ，また，それらの80%が根を形成した．一方，成熟胚を2，4-D 1 μMまたはNAA 5 μMを含む培地に置床すると，成熟胚の90%がカルスを形成した．2，4-D 1 μMを含む培地から形成されたカルス塊を4週間振とう培養すると，カルス塊1個当たり45個体の胚状体が得られ，さらに，これらの胚状体1個体当たり約30本の不定芽が形成された．これらの不定芽は，発根後健全な小植物体に生長し，よく馴化した．これらの結果から，ハマボウフウの増殖には，成熟胚1個から得られたカルスを振とう培養し，誘導した胚状体から不定芽を形成させる方法が，腋芽や成熟胚から不定芽を形成させるより有効であると考えられる．

キーワード：腋芽，再分化植物，成熟胚，セリ科，胚状体，ハマボウフウ．

Glehnia littoralis is a perennial plant belonging to *Umbelliferae*, and is distributed on the coast of Japan, China, Korea and Saghalien islands[8,9]. The young sprouting shoots of *Glehnia* in early spring have been very popular as a good flavored with vegetable since ancient times in Japan. Dried roots of plants are also used as a diaphoretic, an antipyretic and an analgesic medicine in traditional pharmacopoeia in China and Japan[4,6,8,14,16]. Most of farmers have propagated *Glehnia* plants employing blanching culture in Japan. However, the price is etremely expensive because of a short supply with such as culture. Additionally, the wild plants of *Glehnia* are decreasing due to overharvest and very low germinability of seeds[11]. Therefore, we studied the vegetative propagation by tissue culture. It is well known that several plants belonging *Umbelliferae* were easily propagated through somatic embryogenesis and organogenesis from callus culture[1,3,6,7,10,12,17], Recently, Hiraoka and Oyanagi have succeeded in micropropagation of *Glehnia* plants by shoot tip culture[6].

This paper describes a successful propagation of *G. littoralis* by two different methods ; the first, adventitious shoots directly obtained

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from ecised axillary buds, the second, multiple shoots on embryoids indirectly induced through callus cultures from isolated mature embryos.

Materials and Methods

1. Experimental materials

Glehnia littoralis for tissue culture from different explants were collected in the Tokoro area around Lake Saroma in the eastern region of Hokkaido, Japan in 1991. The seeds of *Glehnia* plants were collected from the end of August to the middle of September in 1992 and 1993. The well-dried seeds with pericarp were stored in plastic boxes at room temperature. The seeds collected in 1992 were used for shoot formation from mature embryos. The seeds collected in 1993 were used for suspension culture of callus derived from mature embryos.

2. Light condition of photo period

Plant samples for tissue culture were illuminated vertically at 30 cm of distance and laterally at 10 cm of distance using white fluorescence (Mitsubishi-10SRAM, FL40SW) in incubator (Eyelatron, FLI-301NH, Tokyo Rikakikai CO., LTD.).

3. Cultivation period

Until now there are no data about shoot formation from axillary buds and mature embryos and about embryoid induction from mature embryos in *G. littoralis*. However, data from pre-experiments show necessity for a long term cultivation for these cultures. Thus, most of cultures in this experiments were carried out for 3-4months.

4. Composition of plant hormone in the media

Plant hormones, 2, 4-dichlorophanoxyacetic acid (2, 4-D), 6-benzylaminopurine (BA) and 1-naphthaleneacetic acid (NAA) and their various combination were used for shoot formation and for induction of suspension cells.

5. Shoot formation from leaf blade, petiole and root

Harvested young seedlings of *Glehnia* were rinsed with water. Leaf-blade, petiole and root of *Glehnia* plants were cut off to about 7 cm length, put into miracloth and washed with liquid soap for 15 min. After that they were rinsed thoroughly. Then, plant samples were soaked in 70% ethanol for 7 sec and stirred in 15% hypochlorite solution (0.75% active chlorine) for 20 min. Samples were rinsed 3 times with distilled water. Five millimeter square pieces of leafblade, 7 mm length of petioles or roots were used for shoot formation. Eight segments of tissue were placed in a Petri dish (90 mm) containing 30 m*l* of agar-solidified Murashige and Skoog[15] (MS) medium. The Petri dishes were incubated at 25°C, in 12h photo period. Eplants were transferred every 2 weeks into fresh medium. Three independent

Table 1. Effect of different plant hormones supplimented to MS medium on shoot formation from axillary buds in *G. Iittoralis*.

Plant hormone 2,4-D	BA	NAA (μM)	Shoot number • Axillary bud^{-1} Weels after the start of experiment 4	5	6	7	8	9	10
1	—	—	1±0	3±0	4±0	5±0	5±0	5±0	10±0
1	1	—	1±0	3±0	5±0	5±0	7±0	7±0	9±1
1	5	—	2±0	3±0	5±0	5±0	7±0	7±0	11±1
—	5	5	3±0	4±0	11±1	13±1	21±1	23±1	28±1
—	—	5	2±0	3±0	6±0	6±0	7±0	7±0	13±1
—	3	5	2±0	3±0	9±1	13±1	20±1	23±1	28±1
—	1	5	2±0	4±0	8±1	12±1	18±5	22±1	28±1

Values are means of 3 independent experiments with standard errors. In each experimnt, 8 axillary buds cultured in a Petri dish.

All media were based on MS medium with 3% sucrose and contained various plant hormones. The media were solidified with 0.8% agar.

2,4-D : 2,4-dichrphenoxy acetic acid ; BA : 6-benzylaminopurine ; NAA : potassium 1-naphthalene acetic acid.

experiments were conducted.

6. Shoot formation from axillary buds

Harvested young seedlings were rinsed with water and sterilized by the same method described as the above. Then, explants, 5 mm in length, containing an axillary bud were placed in a Petri dish (90 mm) containing 30 m*l* MS solid medium with various plant hormones (Table 1) and incubated under the same conditions described as the above. Wellrooted plantlets during culture were acclimatized. Three independent eperiments were conducted.

7. Shoot formation from mature embryos isolated from seeds

To sterilize, seeds were put into miracloth, washed with liquid soap for 15 min and rinsed with water. Then, samples were soaked in 70% ethanol for 7s and stirred for 10-30 min in 10-20% hypochlorite solution (0.5-1.0% active chlorine). The seed coat (covering the embryo) was removed and a small hole was made with a needle at the tip of the seeds under a microscope. The seeds were held firmly with forceps and the mature embryos were squeezed out through the hole. Eighteen isolated embryos were placed in each Petri dish (90 mm) containing 30 m*l* MS solid medium with various plant hormones (Table 2). Only for 1st week, embryos were incubated at 25°C in the dark and after that they were incubated at 25°C in 12h photo period for 7 weeks. After that, 18 embryos were transferred into 3 plant boxes (70×70×120 mm) containing 100 m*l* of the same medium. Each box with 6 embryo explants was incubated under the same conditions for 6 weeks. The medium was refreshed every 1 month. The number of shoots and roots appeared on embryo explants were counted at each interval of 2 weeks during experiments which consisted of Petri dish and plant box cultures totally for 11 weeks from the 4th week through 14th week after the start of this eperiment. Well-rooted plantlets during culture were acclimatized. Three independent experiments were conducted.

8. Callus formation from isolated mature embryos

Eighteen mature embryos were placed in each Petri dish (90 mm) containing 30 m*l* of MS solid medium with various plant hormones by the method described as the above. They were placed at 25°C in the dark for only first 2 weeks and for next 2 weeks at 25°C, in 12h photo period. The callus formation was initially appeared from 3rd week after the start of the experiment. Thus, the number of callused explants were counted after 3 weeks culture. After such 4 weeks culture in a dish, callused-explants in 18 embryo explants were transferred into 3 plant boxes (70×70×120 mm), as described above and incubated under the same consitions. The medium was refreshed

Table 2. Effect of different plant hormones supplimented to MS medium on shoot formation from mature embryos in *G. Iittoralis*.

Plant hormone			Shoot number • Embryo explant^{-1}					
2,4-D	BA	NAA	Weels after the start of experiment					
(μM)			4	6	8	10	12	14
1	—	—	1±0	2±1	7±2	15±4	25±6	30±6
1	1	—	0±0	1±1	9±2	11±2	11±2	11±2
1	5	—	0±0	14±4	21±4	37±6	41±13	41±13
—	5	5	1±0	3±1	9±1	15±2	28±3	30±4
—	—	5	4±1	12±0	19±0	19±1	19±1	19±1
—	3	5	2±0	4±1	14±3	20±3	31±4	38±5
—	1	3	1±0	4±1	7±2	12±2	18±3	21±3
—	1	5	1±0	4±1	19±3	24±4	31±4	38±5

Values are means of 3 independent experiments with standard errors. In each experiment, 18 mature embryos were cultured in a Petri dish and they are transferred into 3 plant boxes ater 8 weeks.

All media were based on MS medium described in Table 1.

every 2 weeks. Percentage of callus formation were epressed by number of callused-explants in 18 eplants cultured in plant boxes. Three independent experiments were conducted.

9. Suspension culture of callus derived from mature embryos

Callus formed during 3 weeks to 15 weeks after the start of embryo culture was used in suspension culture. The callus block (about 150×130×80 mm) firmly attached to an embryo explant was well washed 3 times with distilled water. Then, 3 blocks of callus were ground with a mortar and pestle, filtered with 80 μm nylon mesh and suspended in 150 m*l* of hormone-free MS liquid medium. The suspensions were divided into 3 flasks (100 m*l* size) containing each 50 m*l* of suspension. Nine blocks of callus were used in each experiment. Flasks were incubated at 25°C in the dark at 120 rpm on the shaker. After 4-6 weeks, embryoids appeared in flasks were transferred into plant boxes containing MS solid medium. Each plant box containing 12 embryoids were incubated at 25°C in 12h photo period. After 1 month, multiple shoots derived from embryoids were transferred into plant boes containing the same media. After that, the medium was refreshed every 2 weeks for 2 months. Well-rooted plantlets during culture were acclimatized. Three independent eperiments were conducted.

10. Acclimetization of plantlets

The well-rooted plantlets were washed and transferred to vinyl pots (dia. 5 cm, height 7 cm) filled with vermiculite. For acclimatization, the potted plantlets were covered with a plastic cup for 1 week, with a covered tip-off cup for next 1 week and finally uncovered for

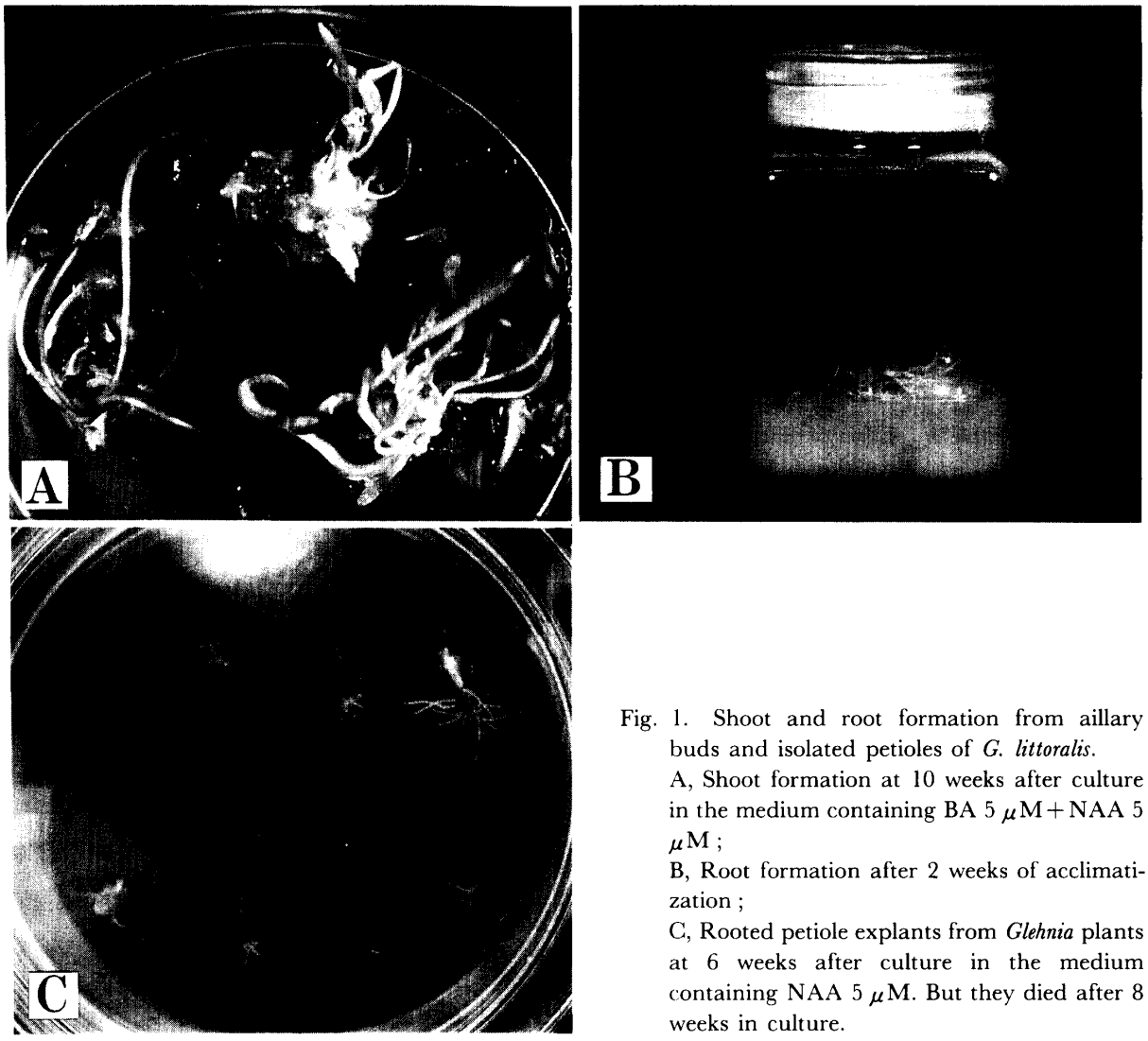

Fig. 1. Shoot and root formation from aillary buds and isolated petioles of *G. littoralis*.
A, Shoot formation at 10 weeks after culture in the medium containing BA 5 μM+NAA 5 μM;
B, Root formation after 2 weeks of acclimatization;
C, Rooted petiole explants from *Glehnia* plants at 6 weeks after culture in the medium containing NAA 5 μM. But they died after 8 weeks in culture.

2 weeks at room temperature. Then, the potted plants were placed in a green house. The plants were supplied with distilled water for 2 weeks when the soil dried and, from the 3rd weeks, they were supplied with Hyponex (source/manufacture) (×1000) at intervals of 3-5 days. Fifty to eighty plantlets were acclimatized in each eperiments. Three independent experiments were conducted.

Results

1. Shoot formation from axillary buds

Only axillary buds showed formation of shoot and root although various parts of plants were tried. Especially, about 30 shoots per axillary bud were obtained on media supplemented with BA (1-5 μM) + NAA (5 μM) after 10 weeks culture (Table 1 and Fig. 1-A) and these shoots also showed good formation of root after transfer to hormone-free MS solid medium (Fig. 1-B). Eighty to ninety percent of plantlets showed good growth after acclimatization (data not shown). In other plant parts, petiole explants formed roots without shoot formation (Fig. 1-C).

2. Shoot and root formation from isolated mature embryos

To obtain higher frequency of shoot forma-

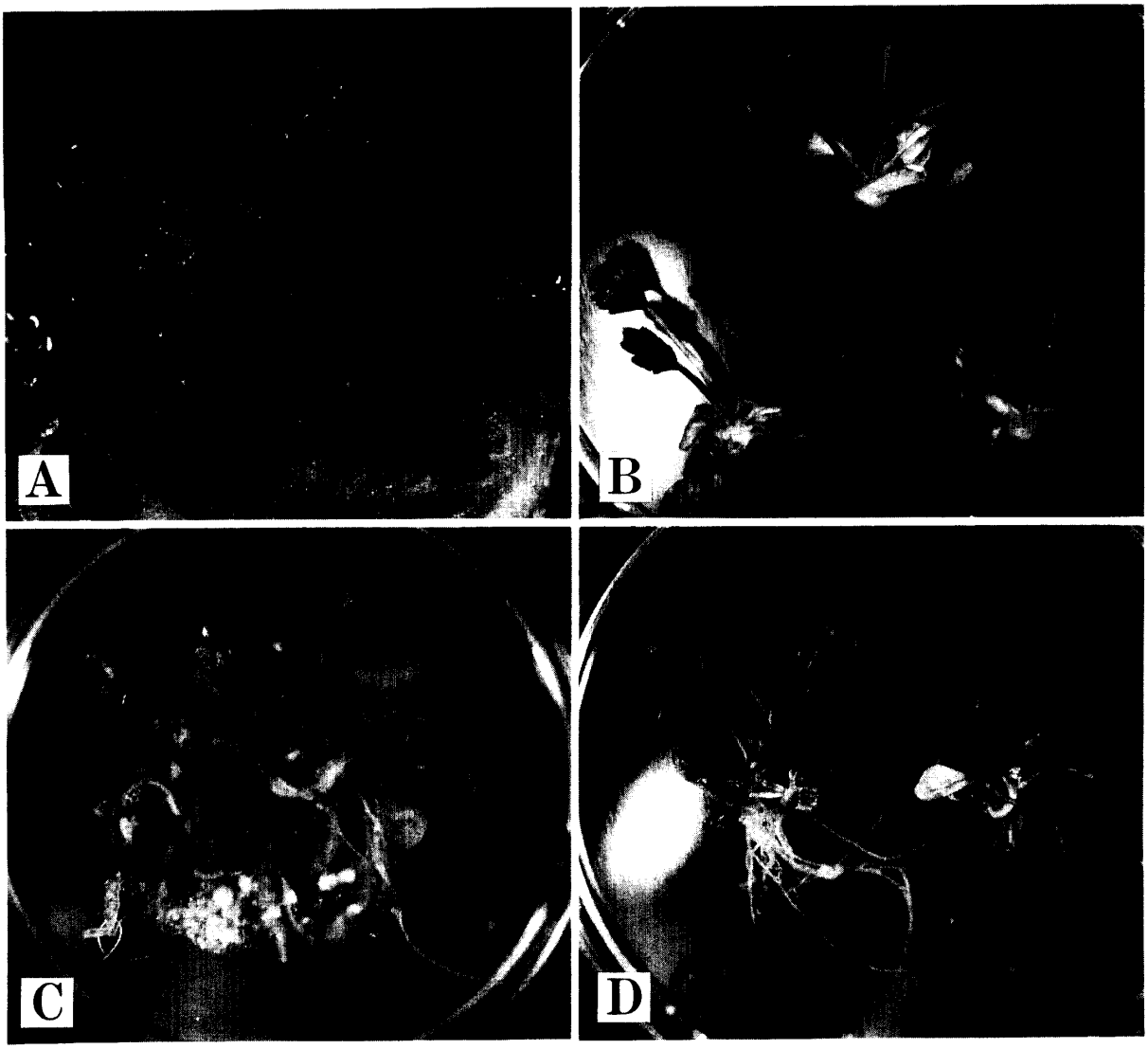

Fig. 2. Shoot and root formation from isolated embryos of *G. littoralis*.
A, Shoot formation after 3 weeks in culture in the medium containing 2, 4-D 1 μM + BA 5 μM;
B, Plantlets without roots after 12 weeks in culture in the medium containing 2, 4-D 1 μM + BA 5 μM;
C, Shoot formation after 8 weeks in culture in the medium containing 2, 4-D 1 μM;
D, Plantlets with firm roots after 12 weeks in culture in the medium containing 2, 4-D 1 μM.

tion than that from axillary bud materials, mature embryos isolated from seeds were used in the next experiment. The medium containing 2, 4-D 1 μM+BA 5 μM showed the highest shoot formation by 41 shoots per embryo (Table 2). The shoot formation in this medium was observed after 3 weeks in culture (Fig. 2-A) but root formation was very scarce (Fig. 2-B). The best formation of shoot and root was obtained from media containing 2, 4-D 1 μM alone and BA 1 μM+NAA 5 μM by 30-40 shoots per embryo (Table 2 and Fig. 2-C) and by 70-80% of rooted embryo explants (Table 3 and Fig. 2-D). Further, 70-95% of plantlets derived from multiple shoots showed good acclimatization (data not shown).

3. Callus formation from isolated mature embryos

The best callus formation from mature

Table 3. Effect of different plant hormones supplimented to MS medium on root formation from mature embryos in *G. Iittoralis*.

Plant hormone			Root formation (%)				
2,4-D	BA	NAA	Weels after the start of experiment				
	(μM)		4	6	8	10	12
1	—	—	0±0	33±17	42±8	50±0	71±15
1	1	—	8±8	25±9	26±9	26±9	26±9
1	5	—	3±1	8±3	14±6	14±6	14±6
—	5	5	0±0	50±15	54±11	54±11	63±7
—	—	5	0±0	8±8	37±9	58±10	65±5
—	3	5	0±0	0±2	15±8	24±6	35±9
—	1	3	0±0	40±23	60±14	60±14	60±14
—	1	5	0±0	33±10	53±2	67±5	77±3

Values are means of 3 idependent experiments with standard errors. In each experiment, 18 mature embryos were cultured in a Petri dish and they were transferred into 3 plant boxes after 8 weeks.
All media were based o MS medium described in Table 1.

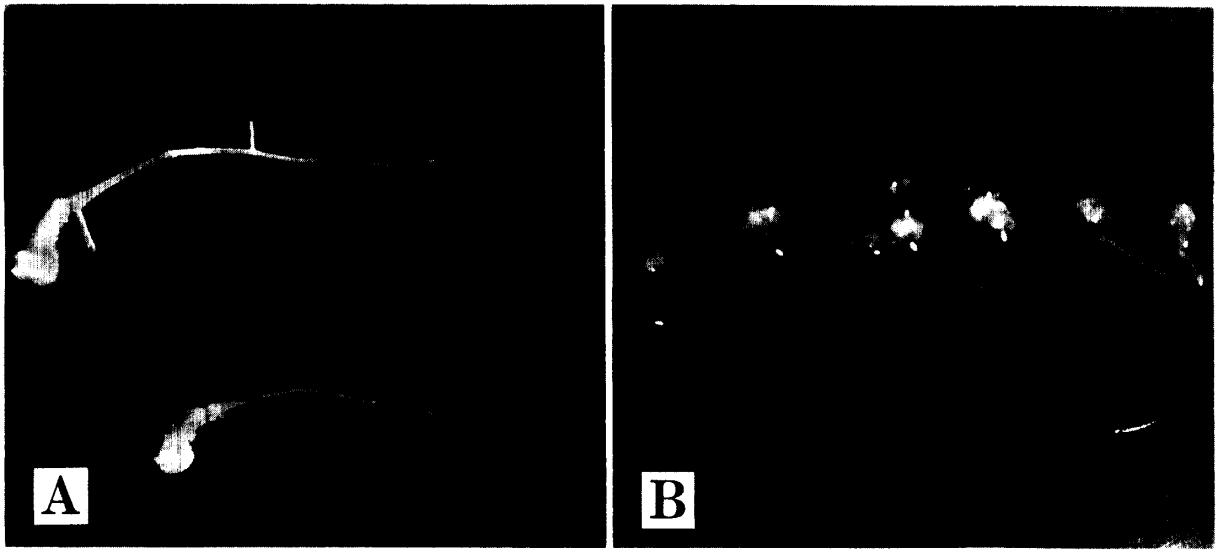

Fig. 3. Embryoid derived from callus blocks by suspension culture.
Callus blocks were cultured in hormone free MS liquid medium for 4 weeks after 10 weeks in culture in the medium containing 2, 4-D 1 μM for cullus formation.
A, Embryoids with long roots;
B, Embryoids with short roots.

embryos was obtained from media containing 2, 4-D 1 μM or NAA 5 μM (Table 4). More than 90% of mature embryos gave rise to a block of firm callus. Firstly after 3 weeks, the calli appeared on embryo explants. At the beginning of culture, calli were firm with right yellow color and they gradually become soft and colored with milky-yellow before 9 weeks. When 1 or 5 μM of BA was added to the medium containing 2, 4-D 1 μM, callus formation was decreased to 30-55%. Less than one percent callus formation was obtained from the medium containing BA 5 μM alone or BA 5 μM plus NAA 5 μM.

Table 4. Effect of different plant hormones supplimented to MS medium on callus formation from mature embryos in *G. littoralis*.

Plant hormone 2,4-D (μM)	BA (μM)	NAA (μM)	Callus formation (%) Weeks after the start of experiment 3	6	9
1	—	—	25±7	85±3	96±2
1	1	—	9±2	40±5	55±7
1	5	—	5±0	16±3	31±6
—	5	5	0±0	1±1	1±1
—	5	—	0±0	0±0	0±0
—	—	5	30±5	90±6	94±3

Values are means of 3 independent experiments with standard errors. In each experment, 18 mature embryos were cultured in a Petri dish and they were transferred into 3 plant boxes after 4 weeks.
All media were based on MS medium described in Table 1.

4. Induction of embryoids in suspension culture

Embryoids with long roots (Fig. 3-A) or short roots (Fig. 3-B) were obtained from a block of firm callus cultured in MS solid media containing 2, 4-D 1 μM, 2, 4-D 1 μM plus 1 or 5 μM BA and NAA 5 μM (Table 5). By suspension culture, calli cultured in the medium containing 2, 4-D 1 μM showed a reliable propagation of embryoids. High frequency of embryoid induction from a block of callus dericed from one matureembryo was obtained from 10-12 weeks callus culture in

Table 5. Effect of different plant hormones supplimented to MS medium on embryoid induction from calli derived from mature embryos in *G. littoralis* by suspension culture.

Period for callus formation (week)	Embryoid number • Callus block^{-1} Plant hormones for callus formation (μM) 2,4-D(1) Type I	2,4-D(1) Type II	2,4-D(1)+BA(1) Type I	2,4-D(1)+BA(1) Type II	2,4-D(1)+BA(5) Type I	2,4-D(1)+BA(5) Type II	NAA(5) Type I	NAA(5) Type II
3	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
4	4±1	0±0	0±0	0±0	0±0	0±0	0±0	0±0
5	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
6	4±1	0±0	0±0	0±0	0±0	0±0	0±0	0±0
7	0±0	3±1	0±0	0±0	0±0	0±0	0±0	0±0
8	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
9	1±1	0±0	0±0	24±6	1±1	1±1	0±0	8±3
10	45±6	10±5	0±0	0±0	0±0	0±0	0±0	4±1
11	12±2	18±9	0±0	0±0	0±0	0±0	1±1	2±1
12	1±1	26±8	0±0	0±0	0±0	0±0	1±1	0±0
13	2±1	16±9	0±0	0±0	0±0	0±0	0±0	0±0
14	2±1	4±4	0±0	0±0	0±0	0±0	0±0	0±0
15	3±1	0±0	0±0	0±0	18±4	17±7	0±0	0±0

Values are means of number of embryoids on 9 flasks with standard errors.
One flask containing cell clusters from 1 callus, which derived from 1 from mature embryo.
Calli fromed during 3 weeks to 15 weeks after the start of embryo culture were used in suspension culture.
Cell clusters in a flask were suspended in 50 ml of hormonefree MS medium and culturd for 4-6 weeks on shaker.
Type I, Embryoids with long roots; Type II, Embryoids with short roots.

Table 6. Effect of different plant hormones supplimented to MS medium on multiple shoot formation from embryoids in *G. littoralis*.

Period for callus formation (Week)	Shoot number•Adventitious embryo^{-1}	
	Plant hormones for callus formation	
	2,4-D(1)	2,4-D(1)+BA(5)
	(μM)	
10	28±4	0±0
11	31±7	0±0
12	19±1	0±0
13	19±1	0±0
15	0±0	23±3

Values are means of 3 independent experiments with standard errors. In each experiment, 12 embryoids derived from calli by suspension culture for 4 weeks were transferred into a plant box containing hormone-free MS solid medium and they were tranferred into 2 plant boxes contaning the same media after 1 month. For shoot formation, embryoids were cultured totally for 2 months.

Fig. 4. Acclimatization of plantlets derived from multiple shoots induced from embryoid.
Callus was induced from embryo explants and cultured for 10-12 weeks in the MS solid medium containing 2, 4-D. Callus blocks were then cultured in hormonefree MS liquid medium for 4 weeks for embryoids formation. The embryoids were cultured in the MS solid medium for formation of multiple shoots and roots for 2 months. Then, plantlets were acclimatized for 1 month.

the medium containing 2, 4-D 1 μM. The highest frequency of induction was about 45 embryoids through a block of callus. Particularly, when the callus was soft and colored with milky-white, high induction of embryoids appeared. The plantlets derived from multiple shoots showed good growth after 1 month of acclimatization (data not shown).

5. Multiple shoot formation from embryoids

Embryoids, which were induced indirectly through callus derived from isolated mature embryos, formed multiple stickshaped shoots after 2 weeks culture in a plant box containing the solid MS medium. The callus block, which cultured for 10 to 11 weeks on the solid medium containing 2, 4-D, showed a high frequency of multiple stick-shaped shoot formation through embryoids. Thereafter, these multiple shoots formed roots and vigorously grew into plantlets. The well-developed shoots were easily separated with finger and calculable accurately. About 30 shoots per embryoid were formed (Table 6). These shoots were also wellrooted during 2 month cultivation on MS solid medium (data not shown). The plantlets derived from multiple shoots showed good growth after 1 month of acclimatization (Fig. 4). About 90% of plantlets showed good acclimatization (data not shown). Consequently, a callus block from 1 mature embryo produced about 65 embryoids.

Discussion

The growth period of *Glehnia* plants in Hokkido is very short. Additionally, it is very difficult to obtain fresh materials of *Glehnia* due to declining populations in the habitated area. Thus, we tried to mass produce *Glehnia* plants from various plant parts and isolated embryo eplants by using tissue culture technique. In the present eperiment, we tried multiple shoot formation from disks of leaf blade, petiole, root and axillary bud of *G. littoralis* plants using MS medium containing various plant hormones. However, only shoot formation from axillary buds was successfully obtained showing about 30 shoots per axillary

bud in the medium containing NAA and NAA+BA.

Miller and Chandler[13] succeeded in producing multiple shoots from cotyledon explants of mature strawberry achenes cultivated in media containing 2, 4-D, BA, NAA and their combinations. In *G. litoralis*, about 40 shoots/embryo (80% of the shoots rooted) were formed in the medium containing BA 1 μM+NAA 5 μM by mature embryo culture. On the other hand, stem and petiole segments of fennel[7,10], or explants of celery[1,2] and carrot[3,12,17] easily produced embryogenic callus when cultivated on medium containing 2, 4-D or NAA. For callus formation from isolated mature embryos o *G. littoralis*, the media containing 2, 4-D 1 μM or NAA 5 μM were also best, about 90% of explants formed block of callus. Further, *Glehnia* callus induced from mature embryo explants, which cultured on a medium containing 2, 4-D, easily gave rise to the well-established suspensions when grown in a shaken liquid medium. In fact, 45 embryoids were produced in suspension culture of callus, which originated from single isolated embryo, for 10 weeks culture in the MS solid medium containing 2, 4-D. Further, all embryoids cultured in the MS solis medium were formed about 30 shoots with roots on each embryoid and these shoots with roots grew into plantlets. Thus, in this culture method, plantlets are able to be produced throughout the year under the controlled condition. Furthermore, most of plantlets showed good growth after acclimatization.

The results show that induction of multiple shoots derived from embryoids suggests an effective method to mass produce *Glehnia* plants compared to formation of multiple shoots from axillary buds or mature embryos. Finally, suspension culture of *Glehnia* callus induced from isolated mature embryos can be advantageous for mass production without any limitation of growth period if this procedure were to be reliable and cost-effective.

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* In Japanese with English summary.
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