Micropropagation of *Diplazium nipponicum*©

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A micropropagation procedure using green globular body (GGB) as an intermediate propagule for *Diplazium nipponicum* Tagawa, an edible fern endemic to Japan, is presented. GGBs were induced from rhizome segments on half-strength Murashige-Skoog (½ MS) medium supplemented with 1 mg·L^{-1} benzyladenine (BA), 20 g·L^{-1} sucrose, and 8 g·L^{-1} agar (pH 5.2). The GGBs were able to multiply efficiently on the full-strength MS medium supplemented with 1 or 2 mg·L^{-1} BA, 20 g·L^{-1} sucrose, and 8 g·L^{-1} agar (pH 5.5). The regeneration of sporophytes from GGB segments was promoted by the addition of 1 mg·L^{-1} 1-naphthaleneacetic acid (NAA) to the full-strength MS medium supplemented with 20 g·L^{-1} sucrose and 8 g·L^{-1} agar (pH 5.5). After acclimatization, the regenerated sporophytes grew normally in a greenhouse.

INTRODUCTION

Diplazium nipponicum Tagawa (Woodsiaceae) is endemic to Japan in the western region (Honshu, Shikoku and Kyushu districts). The sporophyte is evergreen and relatively large size (the entire length of a frond reaches about 1.5 m (Iwatsuki, 1992). This fern has been cultivated as one of the edible wild plants in Fukuoka Prefecture, Kyushu district. The coiled young fronds, which are usually known as croziers or fiddleheads, are harvested up to an elongation of about 20 cm from the soil surface and are packed for shipping after the washing and removal of all black scales. At present, this fern is usually propagated by division. However, the multiplication efficiency is very low.

Murashige (1974) described the availability of adventitious shoot multiplication as the commercial mass propagation system for ferns. However, the system requires great effort and skill for division and subculture of multiplied adventitious shoots. Previously, we have proposed a simple micropropagation system which consists of two processes, that is, the first process of green globular body [GGB, defined by Higuchi et al. (1987)] induction and multiplication and the second process of the sporophyte regeneration. The GGB induction from rhizome segments and its multiplication are able to occur on a medium containing benzyladenine (BA). The regeneration of sporophytes from GGB segments is able to be achieve on a BA-free medium (Amaki and Higuchi, 1991). Cutting and subculture of the GGB segments are very easy because of the globular form of GGB simplifies the precise operation of cutting and inoculation to the medium. This simple micropropagation system was established through the series of tissue culture experiments for nine fern species (Higuchi et al., 1987; Higuchi and Amaki, 1989; Amaki and Higuchi, 1991; Amaki, 1997). In this paper, we report applying the micropropagation method using GGB to *D. nipponicum*.

MATERIAL AND METHODS

Initial Culture of Rhizome Explant and Multiplication of GGB. Rhizomes (about 2 cm long) were prepared from *D. nipponicum* sporophytes cultivated in Fukuoka prefecture. The rhizome tips were washed with a detergent and then rinsed under tap water for 20 min. The tips were immersed in 70% ethanol for 5 min and then 1% NaClO for 15 min. After washing in sterilized distilled water three times, the tips were dissected to cubic explants (4-mm-long sides). Those explants were inoculated on a medium for the induction of GGB [½ Murashige and Skoog (1962) medium (MS) + 1 $mg \cdot L^{-1}$ benzyladenine (BA) + 20 $g \cdot L^{-1}$ sucrose + 8 $g \cdot L^{-1}$ agar (pH 5.2)]. After about 3 months of culture GGBs were obtained. They were subcultured every 3 months on the same medium except that the concentration of BA was modified to 2 $mg \cdot L^{-1}$.

Effects of Medium Constituents on GGB Multiplication and Organogenesis. For the determination of basal medium for GGB multiplication, MS medium strength (1/1, 1/2, 1/3, 1/4, 1/8), sucrose concentration (0, 20, 30, 40, 60, 80 $g \cdot L^{-1}$), and initial pH of medium (4.5, 5.0, 5.5, 6.0, 6.5) were examined. In addition, the effects of various concentrations of BA and 1-naphthaleneacetic acid (NAA) on organogenesis from GGB explants were also examined. The medium [1/1 MS + 20 gL^{-1} sucrose + 8 $g \cdot L^{-1}$ agar (pH 5.5)] was supplemented with each combination of BA and NAA at the concentrations of 0, 0.5, 1.0, 1.5, 2.0 $mg \cdot L^{-1}$.

In all experiments, 10 GGB cubic explants (4 × 4 × 4 mm) were cultured at 24±2 °C and 16-h light with cool-white-fluorescent lamps (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD) / 8-h dark for 3 months. Each GGB segment was put on 10 ml of each medium in a test tube (ϕ20 × 120 mm). Before inoculation of explants, all media poured into test tubes which were closed with aluminum foil were autoclaved at 120 °C for 15 min. After 3 month culture, the numbers and fresh weights of fronds and roots produced on respective GGB explants were recorded.

RESULTS AND DISCUSSION

Rhizome tip explants of *D. nipponicum* produced GGB the same as the other nine-fern species (Higuchi et al., 1987; Higuchi and Amaki, 1989; Amaki and Higuchi, 1991; Amaki and Higuchi, 1992 a,b; Amaki, 1997). The GGBs were multiplied and maintained on the medium containing 2 $mg \cdot L^{-1}$ of BA. The maximum multiplication of GGBs was obtained on the medium containing full strength MS, 20 $g \cdot L^{-1}$ of sucrose, 2 $mg \cdot L^{-1}$ of BA, and pH of 5.5 (Tables 1, 2, 3). The addition of BA markedly inhibited the frond and root formation from GGB segments. The formation of sporophytes (fronds + roots) was promoted by the addition of 0.5 and 1.0 $mg \cdot L^{-1}$ of NAA to BA-free medium. The maximum frond growth was obtained at 1.0 $mg \cdot L^{-1}$ of NAA (Table 4). As mentioned above, the sporophyte of *D. nipponicum* can be easily and quickly propagated using the micropropagation system utilizing GGB subculture after slight modification. The GGB multiplication system has additionally been shown by several other researchers to be useful for multiplying the additional following fern species: *Blechnum spicant* (Fernadez et al., 1996), *Matteuccia struthiopteris* (Iuchi et al., 1999), *Polypodium cambricum* (Bertrand et al., 1999). In addition, GGB induction from rhizome tip segment of *Microsorum musifolium, Drynaria quercifolia*, and *Cyathea lepifera* has been observed in preliminary experiments by our group. These ferns also could be mass propagated by the GGB multiplication system.

Table 1. Effects of medium strength on the growth and organogenesis of green globular body (GGB) explants.

Medium strength	Fresh weight (g/explant)			No. of fronds per explant	No. of roots per explant
	GGB	Fronds	Roots		
1/1	1.38±0.13	1.17±0.17	0.23±0.04	21.3±2.6	40.3±4.8
1/2	1.23±0.16	0.37±0.11	0.13±0.06	4.4±1.5	12.9±4.4
1/3	1.03±0.04	0.07±0.03	0.13±0.07	1.0±0.4	12.9±5.8
1/4	0.73±0.06	0.01±0.01	0.03±0.01	0.1±0.1	4.7±1.7
1/8	0.15±0.01	0.00±0.00	0.00±0.00	0.0±0.0	0.0±0.0

Data were collected after 3 month culture. Means ± SE. n = 10.

Table 2. Effects of sucrose concentration on the growth and organogenesis of green globular body (GGB) explants.

Sucrose ($g \cdot L^{-1}$)	Fresh weight (g/explant)			No. of fronds per explant	No. of roots per explant
	GGB	Fronds	Roots		
0	0.10±0.01	0±0	0.00±0.00	0±0	0.0±0.0
20	1.62±0.12	0±0	0.02±0.01	0±0	2.7±1.4
40	0.88±0.15	0±0	0.01±0.01	0±0	1.2±0.4
60	0.42±0.07	0±0	0.01±0.01	0±0	0.8±0.5
80	0.21±0.02	0±0	0.00±0.00	0±0	0.0±0.0

Data were collected after 3 month culture. Means ± SE. n = 10.

Table 3. Effects of medium initial pH concentration on the growth and organogenesis of green globular body (GGB) explants.

Medium pH	Fresh weight (g/explant)			No. of fronds per explant	No. of roots per explant
	GGB	Fronds	Roots		
4.5	1.14±0.13	0.02±0.01	0.20±0.01	0.2±0.2	2.4±0.8
5.0	1.23±0.23	0.02±0.01	0.10±0.01	0.4±0.3	2.0±1.1
5.5	1.26±0.12	0.00±0.00	0.10±0.01	0.0±0.0	2.0±1.5
6.0	1.16±0.18	0.01±0.00	0.63±0.03	0.2±0.2	8.6±4.1
6.5	1.21±0.06	0.01±0.00	0.07±0.01	0.6±0.6	1.4±0.2

Data were collected after 3 month culture. Means ± SE. n = 10.

Table 4. Effects of BA and NAA on the growth and organogenesis of green globular body (GGB) explants.

PGRs ($mg \cdot L^{-1}$)		Fresh weight (g/explant)			No. of fronds per explant	No. of roots per explant
BA	NAA	GGB	Fronds	Roots		
0	0	1.66±0.14	0.28±0.05	0.09±0.03	5.6±1.8	14.3±2.5
	0.5	1.06±0.05	2.31±0.26	0.91±0.06	28.1±3.2	59.3±4.8
	1.0	0.86±0.18	2.64±0.29	0.78±0.09	28.3±3.4	56.7±7.3
	1.5	1.21±0.06	2.23±0.13	0.79±0.08	25.2±2.9	48.3±5.8
	2.0	1.01±0.11	1.66±0.24	0.80±0.08	18.7±2.8	46.3±5.7
0.5	0	1.71±0.15	0.18±0.04	0.02±0.00	4.0±1.5	2.9±0.7
	0.5	1.48±0.22	0.30±0.07	0.02±0.01	4.7±1.5	3.6±1.3
	1.0	1.87±0.12	0.53±0.08	0.04±0.02	7.0±1.3	6.6±2.3
	1.5	1.82±0.14	0.10±0.03	0.01±0.00	2.6±0.9	2.1±0.7
	2.0	1.68±0.24	0.31±0.07	0.07±0.03	5.4±1.4	6.8±2.3

1.0	0	1.87±0.18	0.12±0.06	0.01±0.00	1.3±0.9	1.4±1.3
	0.5	1.55±0.16	0.08±0.04	0.02±0.00	1.6±0.8	1.2±0.7
	1.0	1.86±0.24	0.06±0.02	0.01±0.00	0.9±0.5	0.6±0.2
	1.5	1.83±0.08	0.08±0.03	0.01±0.00	1.9±0.9	0.9±0.5
	2.0	1.74±0.11	0.16±0.09	0.02±0.00	1.2±0.8	1.8±0.8
1.5	0	2.05±0.17	0.06±0.02	0.01±0.00	0.6±0.2	1.1±0.5
	0.5	1.92±0.22	0.06±0.0.3	0.00±0.00	0.9±0.5	0.8±0.7
	1.0	1.78±0.12	0.08±0.03	0.01±0.00	0.8±0.4	1.1±0.4
	1.5	2.02±0.22	0.14±0.06	0.02±0.01	2.3±1.0	1.9±1.1
	2.0	1.54±0.14	0.07±0.03	0.01±0.01	1.0±0.7	1.2±0.8
2.0	0	2.12±0.11	0.03±0.01	0.01±0.01	1.6±0.6	1.4±1.0
	0.5	2.03±0.18	0.12±0.05	0.01±0.00	2.1±1.0	1.7±0.6
	1.0	1.77±0.17	0.05±0.03	0.01±0.01	1.0±0.9	1.8±0.9
	1.5	1.99±0.14	0.11±0.06	0.01±0.00	2.7±1.5	0.9±0.4
	2.0	2.27±0.12	0.12±0.05	0.01±0.01	2.3±0.8	1.8±1.1
ANOVA	BA	**	**	**	**	**
	NAA	NS	NS	NS	NS	NS
	BA×NAA	NS	**	**	**	**

Data were collected after 3 month culture. Means ± SE. n = 10.

Table 5. The detemined medium compositions for the green globular body (GGB) multiplication and the sporophyte regeneration in *Diplazium nipponicum* examined in this report and another nine species.

	GGB multiplication			Sporophyte regeneration		
Species	MS strength	Sucrose (g/L)	BA (mg/L)	MS strength	Sucrose (g/L)	NAA (mg/L)
Diplazium nipponicum	**1/1**	**20**	**1.0**	**1/1**	**20**	**1.0**
Asplenium nidus	1/1	15	0.5	1/1	15	0
Platycerium bifurcatum	1/1	20	0.5	1/1	20	0
Nephrolepis exaltata	1/2	30	1.0	1/2	30	0
Pteris cretica	1/2	15	0.5	1/2	20	0
Pteris ensiformis	1/2	20	0.5	1/2	20	0
Nephrolepis cordifolia	1/4	30	0.5	1/4	30	0
Rumohra adiantiformis	1/1	30	1.0	1/1	30	0.5
Adiantum raddianum	1/2	20	1.0	1/2	20	1.0
Davallia mariesii	1/4	20	0.5	1/4	20	1.0

In comparison to the results of our past experiments with the nine fern species (Table 5), *Diplazium nipponicum* preferred a relatively high strength MS medium as did *Asplenium nidus*, (Higuchi and Amaki, 1989), *Platycerium bifurcatum* (Amaki, 1997), and *Rumohra adiantiformis* (Amaki and Higuchi, 1991). Frond formation was promoted by addition of NAA as observed for *Rumohra adiantiformis, Adiantum raddianum* (Amaki and Higuchi, 1991), and *Davallia mariesii* (Amaki, 1997). When the GGB segments were inoculated on a rockwool cube used as a supporting material for sporophyte regeneration, sporophyte regeneration was observed earlier than in the case using agar. The sporophyte regenerated on the rockwool cube grew up to a transplantable size within 2 months. After acclimatization, the regenerated sporophytes grew normally in a greenhouse.

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