Micropropagation of Flying Spider-Monkey Tree Fern and Crocodile Fern From Rhizome Segments©

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Flying spider-monkey tree fern [*Cyathea lepifera* (J. Sm. ex Hook.) Copel.] and crocodile fern (*Microsorum musifolium* Copel.) were propagated through tissue culture by a procedure using green globular bodies (GGBs) as an intermediate propagule. Green globular bodys 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 5.6). The GGBs of both species were able to multiply on the full strength MS medium supplemented with 1 mg·L^{-1} BA, 20 g·L^{-1} sucrose, and 8 g·L^{-1} agar (pH 5.6). In *Cyathea lepifera*, the regeneration of sporophytes from GGBs 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). Rockwool cube was the best supporting materials for sporophyte regeneration to obtain high quality transplants for pot plant production.

INTRODUCTION

Flying spider-monkey tree fern [*Cyathea lepifera* (J. Sm. ex Hook.) Copel.] is a species of tree fern and endemic to Taiwan, Philippines, and Japan. Young small sporophytes of *C. lepifera* have become a popular ornamental pot plant recently. Crocodile fern (*Microsorum musifolium* Copel.) is a member of the polypodies and endemic to Malaysia, Philippines, Indonesia, and New Guinea. *Microsorum musifolium* has a prominent network of dark veins which looks like crocodile skin, and is very decorative as a pot or basket plant (Jones, 1987).

Murashige (1974) described the availability of adventitious shoot multiplication as the commercial mass propagation system for ferns. Padhya (1987) reported that the meristematic region of *C. gigantea* had the ability to regenerate new adventitious shoots through the tissue culture procedure. However, the system required great effort and skill for division and subculture of multiplied adventitious shoots. Previously, we have proposed a simple micropropagation system (Amaki and Higuchi, 1991) which consists of two processes, that is, the first process of GGB induction and the second process of the sporophyte regeneration. Green globular body induction from rhizome segments and its multiplication are able to occur on a benzyladenine (BA) containing medium. In this paper, we report the micropropagation method of *C. lepifera* and *M. musifolium* using GGBs.

MATERIALS AND METHODS

Initial Culture of Rhizome Explant and Multiplication of Green Globular Bodies. Tips of rhizomes (about 3cm long) were prepared from *C. lepifera* and *M. musifolium* sporophytes. The rhizome tips were washed with a detergent, and

then rinsed under tap water for 20 min. The tips were immersed in 70% ethanol (EtOH) for 5 min, and then 2% NaClO for 10 min. After washing in sterilized distilled water three times, the tips were dissected to cubic explants (4 mm long). Those explants were inoculated on a medium for the induction of GGBs [Amaki, 1997: $^1/_2$ MS + 1 mg·L^{-1} BA + 20 g·L^{-1} sucrose + 8 g·L^{-1} agar (pH5.6)]. After GGBs were obtained, they were subcultured every 3 months on the same medium for maintenance and multiplication.

Effects of Medium Constituents on GGB Multiplication and Organogenesis. For the determination of basal medium for GGB multiplication of respective species, MS medium strength ($^1/_1$, $^1/_2$, $^1/_3$, $^1/_4$, $^1/_8$), sucrose concentration (0, 20, 40, 60, 80 g·L^{-1}), and medium initial pH (4.5, 5.0, 5.5, 6.0, 6.5) were examined. In addition, the effects of various concentrations of BA and NAA on organogenesis from GGB explants were also examined. Each basal medium for *C. lepifera* [$^1/_1$ MS + 20 g·L^{-1} sucrose + 8 g·L^{-1} agar (pH 5.5)] and *M. musifolium* [$^1/_1$ MS + 40 g·L^{-1} sucrose + 8 g·L^{-1} agar (pH 6.0)] was supplemented with each combination of BA and NAA at the concentrations of 0, 1.0, 2.0 mg·L^{-1}. In these experiments, 10 GGB cubic explants (3 mm long) were put on 10 mL of each medium in a glass test tube (ϕ20 × 120 mm) and cultured at 24±2 °C and 16-h light with cool white fluorescent lamps (40 μmol·m^{-2} s^{-1} PPFD) / 8-h dark for 3 months. Before inoculation of explants, all media were poured into test tubes which were closed with aluminum foil and autoclaved at 120 °C for 15 min. After 3 month culture, the numbers and fresh weights of fronds and roots produced on the GGB explants were recorded.

Effects of Supporting Material on the Regeneration and Growth of Sporophyte. For the determination of effective supporting material for sporophyte regeneration from GGB segments, vermiculite (SS-type, Asahi-Kogyo Co., Ltd., Japan), rockwool cube (Grodan X-TRA, Grodania A/S, Denmark), 2 g·L^{-1} gellan gum (Wako Pure Chemical Industries, Ltd., Japan), and 8 g·L^{-1} agar (Kanto Chemical Co., Inc., Japan) were examined. Each of the supporting materials with the medium constituents for sporophyte regeneration (BA-free medium) was put into a glass test tube (ϕ40 × 130 mm) to 30 mL in capacity. Ten GGB cubic explants (3 mm long) were cultured at 24±2 °C and 16-h light with cool white fluorescent lumps (40 μmol·m^{-2} s^{-1} PPFD) / 8-h dark for 3 months. Before inoculation of explants, vermiculite and rockwool cubes in respective test tubes were autoclaved in a dry state at 120 °C for 20 min, and then, autoclaved medium were poured into the test tubes which were closed with aluminum foil. After 3 months of culture, the numbers and fresh weights of fronds and roots produced on the GGB explants were recorded.

RESULTS AND DISCUSSION

Rhizome tip explants of *C. lepifera* and *M. musifolium* produced GGBs the same as other fern species (Amaki, 1997; Amaki and Kadokura, 2010). The GGBs were multiplied and maintained on a medium containing 1 mg·L^{-1} of BA. However, the growth rate of the two ferns was markedly different. Rhizome explants of *C. lepifera* produced GGBs 1 month after inoculation. On the other hands, GGB production from *M. musifolium* explants was observed after more than 3 months from inoculation.

In *C. lepifera,* the maximum multiplication rate of GGBs was obtained on the medium of 1/1 strength of MS, 20 g·L^{-1} of sucrose, pH of 5.5 (Fig. 1) and 2 mg·L^{-1} of BA (Table 1). The addition of BA markedly inhibited the frond and root forma-

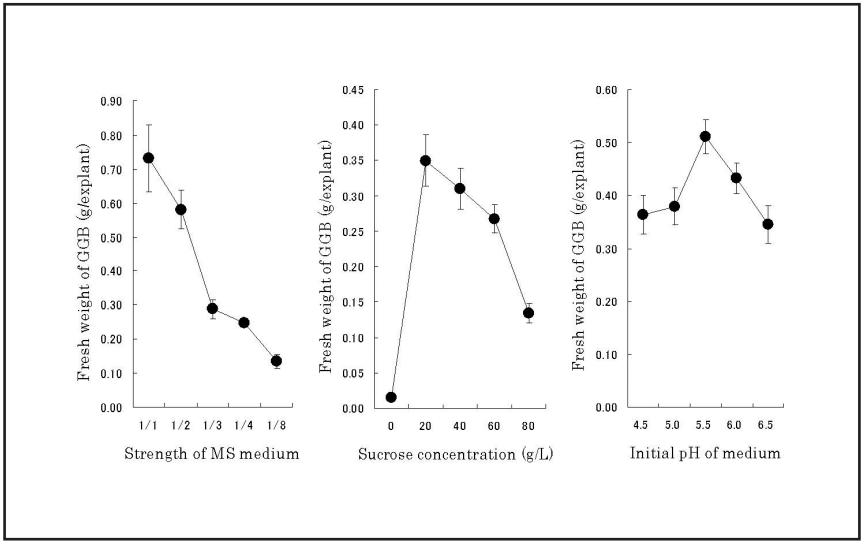

Figure 1. Effects of MS salts strength, sucrose concentration and initial medium pH on the growth of BBG explants in *Cyathea lepifera.*

tion from GGB explants. Frond formation was promoted by the addition of NAA to BA-free medium. The maximum frond growth was obtained at 2.0 $mg \cdot L^{-1}$ of NAA (Table 1). *Cyathea lepifera* showed the same response to NAA as *Rumohra adiantiformis, Adiantum raddianum, Davallia mariesii* (Amaki, 1997) and *Diplazium nipponicum* (Amaki and Kadokura, 2010). In comparison with supporting materials for the sporophyte regeneration, the best growth of fronds and roots from GGB segments was observed on agar and/or gellan gum (Table 2). However, the start of sporophyte regeneration was observed earlier when the GGB segment was inoculated on the rockwool cube than on agar and gellan gum. The sporophyte regenerated on the rockwool cube grew up to transplantable size within 2 months. From the above results the rockwool cube is the best supporting material for sporophyte regeneration among materials used in this experiment, because of rapid regeneration and growth of sporophyte and labor saving for acclimatization procedure. After acclimatization, the regenerated sporophytes grew normally in a greenhouse.

For *M. musifolium* the maximum growth rate of GGBs was obtained on the medium of $^1/_1$ MS medium + 40 $g \cdot L^{-1}$ of sucrose (Fig. 2). Experiments on effects on the initial medium pH, BA and NAA concentration on GGB growth and organogenesis are now in progress because of the slow growth rate of *M. musifolium* GGBs compared with that of *C. lepifera.*

Table 1. Effects of BA and NAA on the growth and organogenesis from GGB explants in *Cyathea lepifera*. (n = 10. Values in table are means ± SE.)

Concentration (mg/L)		Fresh weight (g/explant)			No. of fronds	No. of roots
BA	NAA	Fronds	Roots	GGB	per explant	per explant
0	0	2.51±0.29	0.15±0.05	0.23±0.02	36.4±5.45	22.4±4.43
	1.0	2.60±0.38	0.29±0.04	0.27±0.03	36.8±3.98	25.0±2.57
	2.0	2.80±0.14	0.35±0.06	0.33±0.02	47.6±1.44	23.8±2.84
1.0	0	0.00±0.00	0.00±0.00	0.28±0.01	0.00±0.00	0.00±0.00
	1.0	0.00±0.00	0.00±0.00	0.29±0.02	0.00±0.00	0.00±0.00
	2.0	0.00±0.00	0.00±0.00	0.35±0.04	0.00±0.00	0.00±0.00
2.0	0	0.00±0.00	0.00±0.00	0.11±0.01	0.00±0.00	0.00±0.00
	1.0	0.00±0.00	0.00±0.00	0.19±0.03	0.00±0.00	0.00±0.00
	2.0	0.00±0.00	0.00±0.00	0.30±0.03	0.00±0.00	0.00±0.00

Table 2. Effect of supporting material on the regeneration and growth of sporophytes from GGB segment.

	Fresh weight (g/explant)			No. of fronds	No. of roots
Supporting material	Fronds	Roots	GGB	per explant	per explant
Vermiculite	0.18±0.05	0.00±0.00	0.41±0.12	12.8±3.88	0.4±0.22
Rockwool cube	0.63±0.08	0.12±0.04	0.93±0.09	72.7±9.94	19.2±5.06
Gellan gum (2g/L)	3.15±0.38	0.20±0.07	1.00±0.14	119.5±12.7	40.3±8.79
Agar (8g/L)	3.51±0.14	0.36±0.04	1.70±0.13	138.3±6.48	72.5±7.83

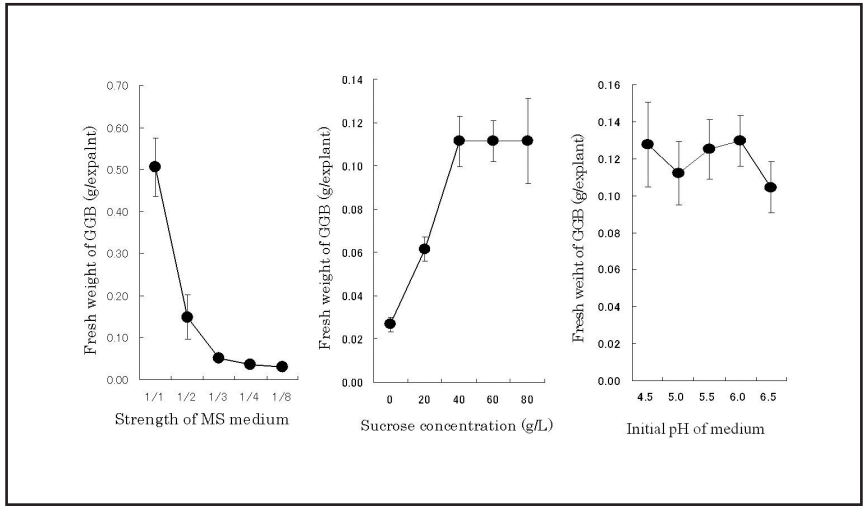

Figure 2. Effects of MS salts strength, sucrose concentration and initial medium pH on the growth of BBG explants in *Microsorum musifolium*.

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