Micropropagation of Ornamental Aquatic Plants, *Glossostigma*, *Microcarpaea*, and *Limnophila*©

Toru Niki and Wakanori Amaki
Department of Agriculture, Tokyo University of Agriculture, 1737 Funako, Atsugi, Kanagawa 246-0034, Japan
Email: amaki@nodai.ac.jp (corresponding author)

Three aquatic plants, *Glossostigma elatinoides* (Benth.) Hook.f., *Limnophila* sp. (unidentified), and *Microcarpaea minima* (K.D. Koenig ex Retz.) Merrill. were examined to clarify the optimal culture medium and cultural conditions for their micropropagation. Murashige and Skoog (MS) medium strength (1/1, 1/2, 1/4 and 1/8), sucrose concentration (0, 10, 20, 30, and 40 g·L^{-1}), initial pH of medium (4.5, 5.0, 5.5, 6.0, and 6.5), and the kind and concentration of gelling agent (6, 7, 8, 9, 10 g·L^{-1} for agar and 3 g·L^{-1} for gellan gum) were examined. The optimal medium conditions for all of the examined plants was ½ MS, 20 g·L^{-1} sucrose, and 3 g·L^{-1} gellan gum. The optimal medium pH was 5.0 for *G. elatinoides* and 6.0 for *M. minima* and *Limnophila*. Water supplement during culture was effective on the growth and acclimatization of multiplied plants. The optimal timing of supplement was after 20 days for *G. elatinoides* and 30 days for *M. minima* and *Limnophila* from the explant inoculation.

INTRODUCTION

In recent Japan, the enjoyment of aquariums is booming, and the commercial demand for aquatic plants has greatly increased. In vitro tissue culture has been identified as an effective technique for large scale multiplication of elite plants. Several reports have demonstrated that aquatic plants can be multiplied by in vitro propagation through proliferation from pre-existing bud in *Cryptocoryne*, *Anubias*, *Myriophyllum*, and *Potamogeton* (Kane et al., 1990, 1999; Huang et al., 1994; Zhou et al., 2006; Kanchanapoom et al., 2012), through adventitious shoot formation in *Limnophila* and *Aponogeton* (Rao and Mohan Ram, 1981; Carter and Gunawardena, 2011) or embryogenesis in *Scirpus* and *Nymphoides* (Wang et al., 2004; Myung et al., 2010). However, the most suitable micropropagation method for each species which are commercially common in Japan is still uncertain. That is, the present conditions for the mass production of high-quality aquatic plants for aquarium is not known. Therefore, we started a series of experiments to clarify the basic culture conditions for mass production of aquatic plants by micropropagation. At first, we selected three aquatic plants for experiment materials because those plants are used for the aquarium frequently in Japan. We chose the pre-existing bud culture method because we wanted to propagate clone plants without somatic variations. In this reports, we tried to clarify the most suitable culture medium and the effect of water supplement during in vitro culture for the acclimatization to aquarium conditions.

MATERIALS AND METHODS

Preparation of Materials

Shoot tip explants (about 1 cm long) were prepared from in vitro mother plants, *Glossostigma elatinoides* (*Phrymaceae*), *Microcarpaea minima* (*Plantaginaceae*) and *Limnophila* sp. (unidentified; *Plantaginaceae*) which were provided gratis by EARTH ONE Ltd. (Tokyo, Japan). The explants were placed on the multiplication medium [half strength of Murashige and Skoog (1962) medium (MS) + 20 g·L^{-1} sucrose + 8 g·L^{-1} agar, pH 5.8] for maintenance and multiplication of stock plants for the following experiments.

Experiments for the Optimal Medium Constitution (Experiment 1)

Murashige and Skoog medium strengths (1/1, 1/2, 1/4 and 1/8), sucrose concentrations (0, 10, 20, 30, and 40 g·L^{-1}), initial pH values of the media (4.5, 5.0, 5.5, 6.0, and 6.5), and the kind and concentration of gelling agents [6, 7, 8, 9, 10 g·L^{-1} for agar (Kanto Chemical Co.

Inc., Japan) and 3 $g \cdot L^{-1}$ for gellan gum (Wako Pure Chemical Industries, Ltd., Japan)] were examined.

Effects of Water Supplement during Culture (Experiment 2)
Fifty milliliters of autoclaved pure water with the pH adjusted to 5.8 using 0.1 N NaOH just before autoclaving was poured into each test tube on the gelled media at 10, 20, 30 days after the inoculation of the explants. The growth after water supplement was compared with the cultures without water supplement (control).

Culture Conditions and Measurements
Twenty milliliters of each medium was poured into a φ40×130 mm flat-bottomed glass test tube and autoclaved at 120°C for 15 min before explant inoculation. All cultures were incubated under 23±1°C and 16-h light with cool white fluorescent lamps (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD) with an 8-h dark period. Total fresh weight of multiplied plants in each test tube was measured at 40 days for Experiment 1 and 50 days for Experiment 2 after the inoculation of explants.

RESULTS AND DISCUSSION
The results of media strength are presented in Figure 1. On the MS medium used as the standard medium for plant tissue culture abnormal growth (fading and death of leaves, and growth retardation of roots) was observed. The cause of the abnormal growth seems to be high osmotic pressure stress. All three species showed the best growth on the ½ MS medium. Figure 2 shows the results of sucrose concentration. The optimal concentration was 20 $g \cdot L^{-1}$ for *Glossostigma elatinoides*. The highest value of fresh weight in *Microcarpaea minima* and *Limnophila* was obtained on the medium supplemented with 40 $g \cdot L^{-1}$ of sucrose. However, more than 30 $g \cdot L^{-1}$ of sucrose caused the abnormal growth and early fading and death of leaves. The best concentration for the normal growth was also 20 $g \cdot L^{-1}$ in *M. minima* and *Limnophila*. The optimal pH was 5.0 for *G. elatinoides* and 6.0 for *M. minima* and *Limnophila* (Fig. 3). The best gelling agent for all of three species was 3 $g \cdot L^{-1}$ of gellan gum (Fig. 4).

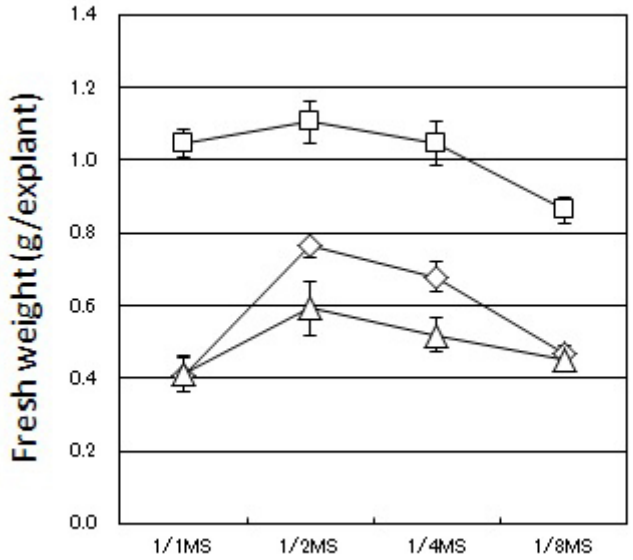

Fig. 1. The effects of medium strength on the growth of three aquatic plants, *Microcarpaea minima* (□), *Glossostigma elatinoides* (◇), and *Limnophila* sp. (△).

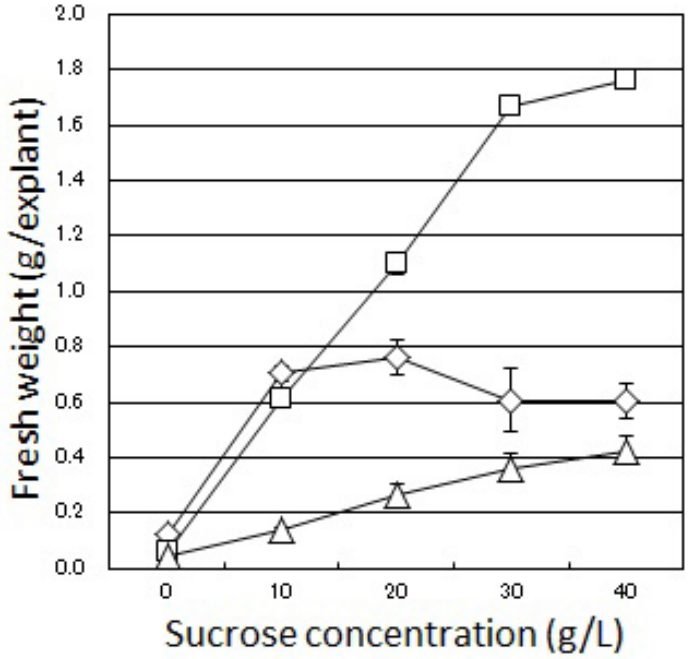

Fig. 2. The effects of sucrose concentration on the growth of three aquatic plants, *Microcarpaea minima* (□), *Glossostigma elatinoides* (◇), and *Limnophila* sp. (△).

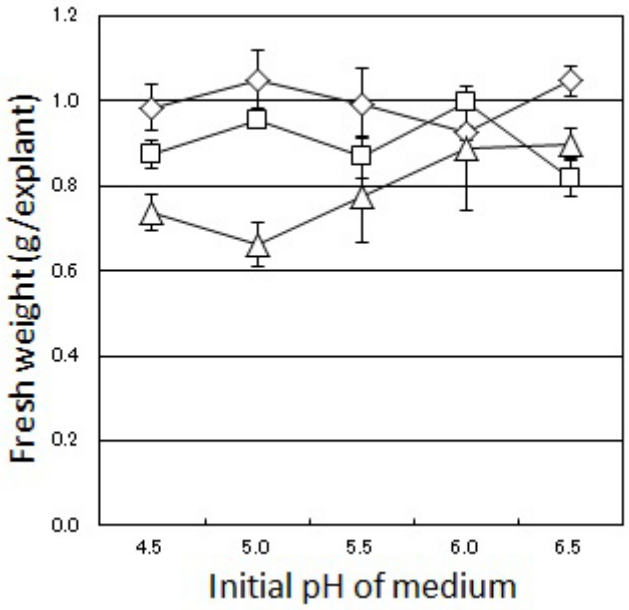

Fig. 3. The effects of initial pH of medium on the growth of three aquatic plants, *Microcarpaea minima* (□), *Glossostigma elatinoides* (◇), and *Limnophila* sp. (△).

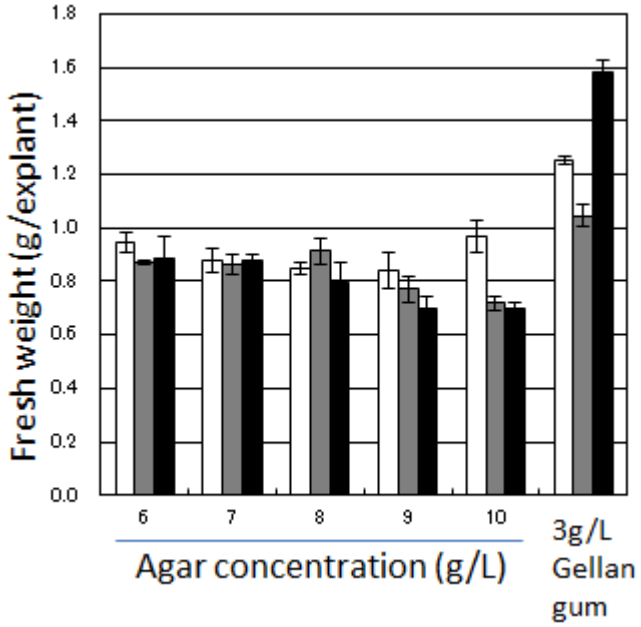

Fig. 4. The effects of gelling agents on the growth of three aquatic plants, *Glossostigma elatinoides* (□), *Microcarpaea minima* (▒), and *Limnophila* sp. (■).

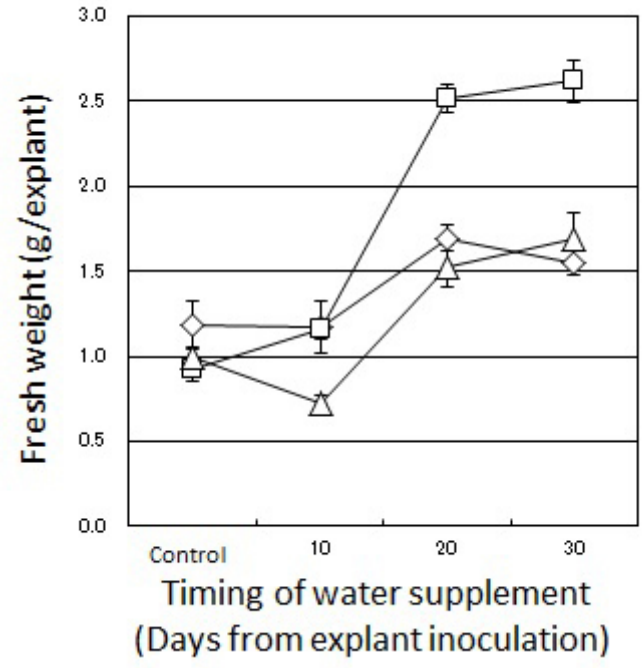

Fig. 5. The effects of water supplement and its timing on the growth of three aquatic plants, *Microcarpaea minima* (□), *Glossostigma elatinoides* (◇), and *Limnophila* sp. (△).

Water supplement during culture was effective on the growth and acclimatization of multiplied plants (Fig. 5). All three plants investigated are amphibious-respondors (amphibious-respondor refers to plant which can live under water and in the ground. It is a term mainly used for the plant groups which can live also at the place where a water level is

fluctuated in the field of ecology) which are able to grow in water presence or absence condition. After water supplement, leaves of the plants were morphologically changed from aerial (emerged) leaves to submerged leaves (leaves in the water) which were characterized with the linear and thin thickness. Therefore, multiplied plants on the gelled media with gellan gum could be acclimatized to aquarium conditions by water supplement during culture. The optimal timing of supplement was after 20 days for *G. elatioides* and 30 days for *M. minima* and *Limnophila* from the explant inoculation (Fig. 5).

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