Micropropagation of ornamental aquatic plants, *Glossostigma*, *Microcarpaea* and *Limnophila* 2. Effect of $CaCl_2 \cdot 2H_2O$, KH_2PO_4, Fe-EDTA concentrations on the growth of explants©

M. Minamiyama, A. Noguchi and W. Amaki[a]

Department of Agriculture, Tokyo University of Agriculture, 1737 Funako, Atsugi, Kanagawa 246-0034, Japan.

Abstract

For each concentration of $CaCl_2 \cdot 2H_2O$ (1.5, 0.6 and 0.3 mM), KH_2PO_4 (0.63, 0.25, and 0.13 mM), and Fe-EDTA (50, 25, and 13 µM) in the tissue culture medium, the effects on the in vitro growth of three aquatic plants, *Limnophila* sp. (unidentified), *Glossostigma elatinoides* (Benth.) Hook.f., and *Microcarpaea minima* (K.D. Koenig ex Retz.) Merrill., were examined. On the result of $CaCl_2 \cdot 2H_2O$, *Limnophila* and *M. minima* showed the highest value of plant fresh weight (FW) on medium supplemented at 0.3 mM. However, leaf yellowing and abnormal growth occurred at 0.3 mM in *Limnophila*. On the other hand, leaf color of M. minima became darker at the lower concentration. In *G. elatinoides*, the highest value of FW was obtained when the concentration was 0.6 mM. In all three species, lowering the concentration of KH_2PO_4 decreased the FW of plants. There was a clear tendency for FW to increase with decreasing Fe-EDTA concentration in *Limnophila* and *M. minima*. On the other hand, FW was maximized at 25 µM of Fe-EDTA and when the concentration was lowered to 13 µM, FW remarkably decreased in *G. elatinoides*.

INTRODUCTION

In recent years in Japan, the commercial demand for tissue cultured aquatic plants has considerably increased. There are already several reports on the growth of aquatic plants by tissue culture (Rao and Ram, 1981; Huang et al., 1994; Kane et al., 1999; Zhou et al., 2006; Kanchanapoom et al., 2012; Jabir et al., 2016). We have also reported on optimized conditions of medium for three aquatic plants, *Glossostigma elatinoides* (Benth.) Hook.f., *Limnophila* sp. (unidentified), and *Microcarpaea minima* (K.D. Koenig ex Retz.) Merrill (Niki and Amaki, 2014). That is, the optimal strength of the Murashige and Skoog (1962) medium (MS medium) was half strength, and the optimal concentrations of sucrose and gellan gum were 20 and 3 g L^{-1}, respectively. The optimum pH value at the time of medium preparation was 5.0 for *G. elatinoides* and was 6.0 for *Limnophila* sp., and *M. minima*. With these medium conditions, in vitro propagation of three aquatic plants became possible, but leaf yellowing and withering occurred after 2 months culture. The cause might be expected to be an imbalance in constituents of the medium or excess and/or deficiency of specific constituents. Considering the natural environment of the three plants' habitat, there was a possibility that the ½ MS medium concentration was too high. In this report we investigated the effects of lowering $CaCl_2 \cdot 2H_2O$, KH_2PO_4, and Fe-EDTA concentrations on the growth of three aquatic plants [*G. elatinoides* (Benth.) Hook.f., *Limnophila* sp. (unidentified), and *M. minima* (K.D. Koenig ex Retz.) Merrill].

MATERIALS AND METHODS

Preparation of materials

Shoot tip explants (about 1 cm long) were prepared from in vitro mother plants, *G. elatinoides* (*Phrymaceae*), *Microcarpaea minima* (*Plantaginaceae*) and *Limnophila* sp.

[a]E-mail: amaki@nodai.ac.jp

(unidentified; *Plantaginaceae*). The explants were inoculated in the multiplying medium which was ½ strength MS constituents supplemented with 20 g L^{-1} sucrose and 3 g L^{-1} gellan gum (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (pH 5.8) for maintenance and multiplication of stock plants for the following experiments.

Experiments for the optimal concentration of three constituents

With the concentration of ½ MS as the control concentration, the concentrations of respective constituents such as calcium chloride ($CaCl_2 \cdot 2H_2O$) and potassium phosphate monobasic (KH_2PO_4) (Kanto Chemical Co. Inc., Japan) and ferric monosodium ethylene-diaminetetraacetate (Fe-EDTA) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were lowered. The concentrations of $CaCl_2 \cdot 2H_2O$ were 1.5, 0.6 and 0.3 mM; KH_2PO_4 were 0.63, 0.25, and 0.13 mM; and Fe-EDTA was 50, 25, and 13 μM.

Culture conditions and measurements

Thirty mL of each medium was poured into φ 40×150 mm flat-bottomed glass test tubes and autoclaved at 120°C for 15 min before explant inoculation. One explant (shoot tip explant about 1 cm long) was inoculated in a test tube and closed with double layers of aluminum foil. All cultures were incubated under 23±1°C and 16-h light with cool white fluorescent lamps (40 μmol m^{-2} s^{-1} PPFD)/8-h dark condition. Total fresh weight of multiplied plants in each test tube was measured at 60 days after the inoculation of explants.

RESULTS AND DISCUSSION

The results for $CaCl_2 \cdot 2H_2O$ concentrations are presented in Figure 1. *Limnophila* and *M. minima* showed the largest value of fresh weight (FW) per plant on the medium supplemented at 0.3 mM. From visual observation, more roots were at the lower concentration. However, leaf yellowing and abnormal growth occurred at 0.3 mM in *Limnophila*. On the other hand, leaf color of *M. minima* became darker at the lower concentration. In *G. elatinoides*, the highest value of FW was obtained at a concentration of 0.6 mM. In the description that explains the medium components of freshwater aquatic plants (Watanabe, 2012), although the target plants are not specified, the amount of $CaCl_2 \cdot 2H_2O$ is less than 1/10 the concentration of MS in most of the media and there is a possibility that it may be even lowered. As a result of tentative judgment including plant form, the optimum concentration for each plant is 1.5 mM for *Limnophila*, 0.6 mM for *G. elatinoides* and 0.3 mM for *M. minima*.

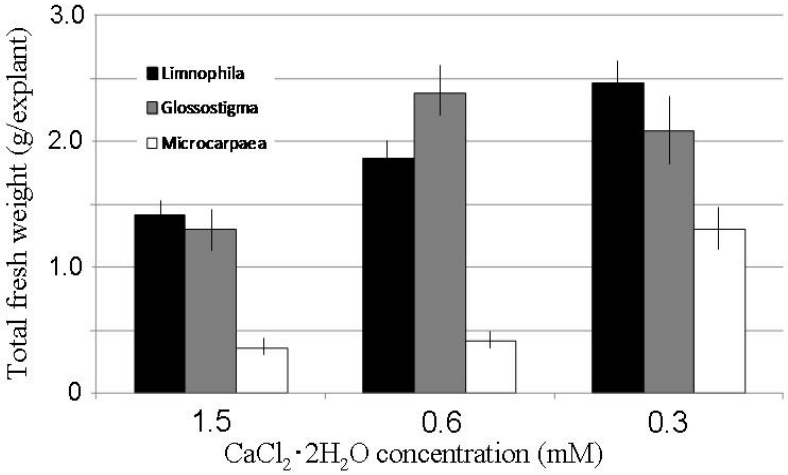

Figure 1. Effect of $CaCl_2$ concentration in the medium on the in vitro growth of plants in *Limnophila*, *Glossostigma*, and *Microcarpaea*. Vertical bars represent the value of standard errors (*n*=5).

Figure 2 shows the results of KH_2PO_4 concentration. In all three species, lowering the concentration of KH_2PO_4 decreased the fresh weight of the plants. In particular, FW of *G. elatinoides* and *M. minima* were significantly less than half for them at 0.13 mM when compared with 0.63 mM. Therefore, it was shown that lowering the concentration of KH_2PO_4 to not more than 0.63 mM of the ½ MS is not preferable for maintaining growth of all three species. It is shown that the demand for phosphate is high in some plants (George, 1993), and it seemed that it is necessary to experiment at even higher concentration of phosphate.

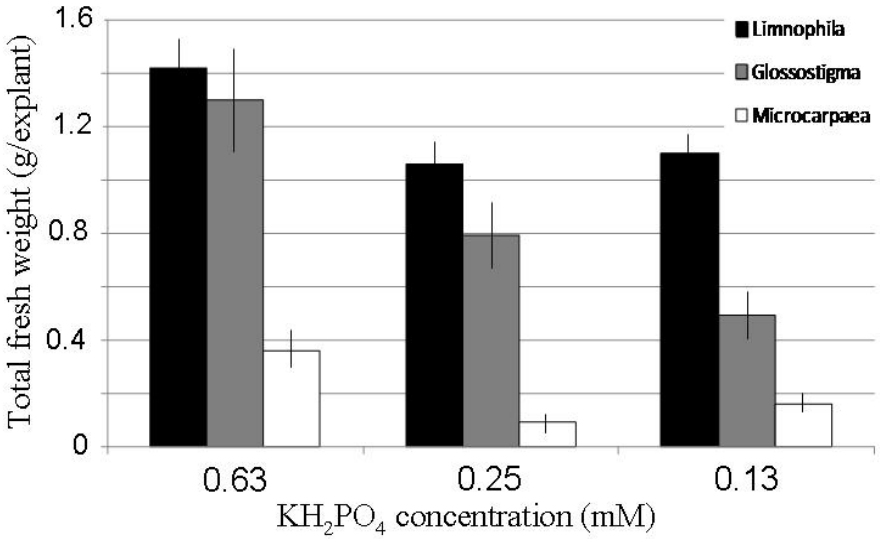

Figure 2. Effect of KH_2PO_4 concentration in the medium on the in vitro growth of plants in *Limnophila*, *Glossostigma* and *Microcarpaea*. Vertical bars represent the value of standard errors (*n*=5).

Figure 3 shows the results of Fe-EDTA concentration. In *Limnophila* and *G. elatinoides*, there was a clear tendency for FW to increase with decreasing Fe-EDTA concentration. On the other hand, FW was maximum at 25 μM, and when the concentration was lowered to 13 μM, FW remarkably decreased in *M. minima*. In considering this result, it is necessary to consider that Fe-EDTA is composed of Fe as an essential micronutrient element and EDTA as a chelating agent having a growth inhibiting effect in some case (Legrand, 1975; Dalton et al., 1983). Watanabe (2012) listed a medium suitable for cultivating many freshwater aquatic plants. The amount of Fe in the medium is 20 μM, which is consistent with the results of our experiment in *Limnophila* and *G. elatinoides*. In addition, both species may be more susceptible to EDTA toxicity. However, *M. minima* showed exactly the opposite tendency. *Microcarpaea* minima is endemic to India and Southeast Asia, and it is said to grow vigorously in paddy fields and river floodplain (Ishida et al., 2010). In *M. minima*, Fe demand is expected to be high compared with other two species. In order to confirm, it is necessary to conduct the experiment by changing the concentration of EDTA alone, which is a future research subject.

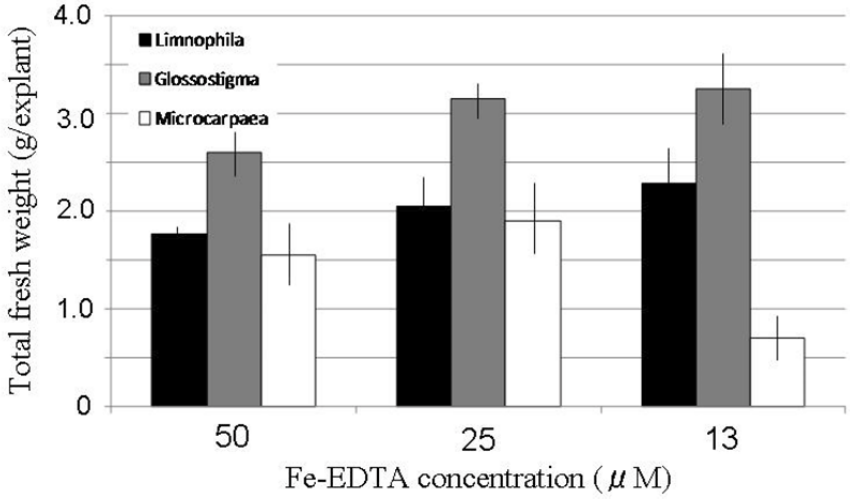

Figure 3. Effect of Fe-EDTA concentration in the medium on the in vitro growth of plants in *Limnophila*, *Glossostigma* and *Microcarpaea*. Vertical bars represent the value of standard errors (*n*=5).

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