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OPTIMIZATION OF NUTRIENT MEDIA FOR RAPID AND EFFICIENT MICROPROPAGATION OF *BACOPA MONNIERI* AND *CERATOPHYLLUM DEMERSUM*

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ABSTRACT

Bacopa monnieri L. Pennell, which has an important medicinal value and *Ceratophyllum demersum* L. having a great potential in cleaning water contaminated with heavy metals are also an indispensable plants in aquariums. Due to these features, the production of uniform and healthy plants in abundance in these species is important. On the other hand, it is possible to produce thousands of healthy plants with the same genetic structure in a short time with *in vitro* techniques. In this study, the effects of 12 different basic nutrient media, six different sucrose and seven different agar concentrations were investigated for the rapid and low-cost *in vitro* production of B. *monnieri* and C. *Demersum* species. When shoot formation and rooting rate were evaluated together, it was observed that Schenk ve Hildebrandt (SH) for *C. demersum* and Orchimax basic nutrient media for *B. monnieri* gave better rate of micropropagation than other media tested. In different sugar applications, the highest micropropagation results were obtained from the medium containing 20 g/L sucrose in *B. monnieri* and 50 g/L sucrose in *C. demersum*. In addition, *C. demersum* achieved the highest micropropagation in liquid medium without agar, and *B. monnieri* in medium containing 5 g/L agar. As a result of this study, micropropagation was achieved at high rates in both species without using agar, the most expensive compound of nutrient media, or by using it at low rates. Regenerated plants were transferred to aquariums and achieved 100% adaptation.

Keywords: *In vitro, SH medium, Orchimax medium, agar, sucrose.*

INTRODUCTION

Aquatic plants produce organic compounds such as carbohydrates, lipids, proteins and pigments through photosynthesis using carbon, nutrients and solar energy (Mansour et al., 2022). Therefore, they are the main producers of aquatic environment and maintain the balance between aquatic organism (Özcan et al., 2023). Aquatic plants, which are resistant to several pollutants and can grow in soils that are not suitable for the cultivation of terrestrial plants, have begun to gain great importance in areas such as biofuel, cosmetics, functional foods and pharmaceuticals

(El-Shenody et al. 2019; Mansour et al.2022). Moreover, some aquatic plants play an important role as biological indicators in detecting water pollution and can also be used to clean water and protect shorelines, ponds and lakes from erosion. (Öztürk et al., 2004; Doğan, et al., 2018). Many aquatic plant species are also used as aquarium plants, and in addition to providing a beautiful appearance to aquariums, they also make a significant contribution to the protection and feeding of fish and the improvement of water quality (Özcan et al., 2023).

Bacopa monnieri L. Pennell and *Ceratophyllum demersum* L. species, which have important place in the pharmaceutical industry and phytoremediation of water (Keskinkan et al., 2004; Vishnupriya and Padma, 2017; Abu, 2017), are also indispensable plants in aquariums. These species can be propagated by seeds or cuttings, but these methods are very slow and costly. In addition, genetic changes occur in seed propagation, and the risk of contamination with bacterial and fungal diseases is much higher in propagation with cuttings. On the other hand, it is possible to propagate millions of disease-free plants with the same genetic structure within a year with *in vitro* techniques. For fast and low-cost *in vitro* production, the nutrient media content used for each plant must be optimized. Therefore, various basic nutrient media, different sucrose and agar concentrations were tested for the micropropagation of *B. monnieri* and *C. Demersum* in the current study.

MATERIALS AND METHODS

This study was carried out in the laboratories of Fisheries and Aquaculture and Field Crops Departments, Faculty of Agriculture, Ankara University in 2020 and 2021. In the study, 12 different basic nutrient media developed by different researchers for different plant species were tested for micropropagation of *B. monnieri* and *C. Demersum* (Table 1). Among these media, MS and B_5 vitamins were added to the SH medium. Additionally, 0-60 g/l sucrose and 0-7 g/l agar were added to the best medium determined for each plant species to find the best sugar and agar concentrations. After adjusting the pH of the prepared media to 5.6, they were autoclaved at at 121 °C under 1.4 kg/cm^2 pressure for 20 minutes.

Apical and axillary buds taken from plantlets developed *in vitro* under sterile conditions were cultured on nutrient media in Duchafe Sterivent culture containers. Four buds were placed in each culture container and measurements were made 4 weeks after the start of culture. All experiments were set up in 4 replicates of 4 explants each. The data obtained were subjected to analysis of variance (ANOVA) and the significance between the means were determined with the Tukey test. After four weeks of culture initiation, the plantlets that developed and rooted in the tested nutrient media were transferred to aquariums with white light, where the temperature was 24 °C and the pH was 6.5-7.2.

RESULTS AND DISCUSSION

Effect of different nutrient media

In both species, shoot development started one week after culture initiation. Shoot formation and rooting results obtained from 12 different basic nutrient media used

for micropropagation of *Bacopa monnieri* are given in Table 1. As seen in the Table, the highest number of shoots per explant was obtained from Anderson's Rhododendron nutrient medium with 6.31 (Fig. 1a), while the highest shoot length was recorded on MS medium with 5.94 cm. The lowest number of shoots per explant and shoot length were obtained from MS and MS No: 3B basic nutrient media with 1.44 and 1.12 cm, respectively. In this species, the highest number of roots (14.5 pieces) and root length (7.15 cm) per explant were found in Orchimax medium (Fig. 1b). When the shoot formation and rooting results were evaluated together, it was determined that Orchimax nutrient medium was more suitable for micropropagation of *B. monnieri*.

Among the 12 different nutrient media tested for *in vitro* micropropagation of *C. demersum*, the highest number of shoots per explant was obtained from Knudson C Orchid medium with 10.25 and the highest shoot length of 4.80 cm (Fig. 1c, Table 2). However, the rooting rate was quite low in this medium. The lowest shoot number and shoot length per explant were recorded in CHU (N6) nutrient medium, with 3.0 and 2.17 cm, respectively (Table 2). While the highest number of roots per explant was obtained from Orchimax (12.25) and SH (11.50) nutrient media, the highest root length was found in SH + MSvit medium, with 2.97 cm (Fig. 1d). When all the results were evaluated, it was determined that the SH medium was the most suitable basic nutrient medium for the micropropagation of *C. demersum* (Table 2).

Table 1. Effect of different basal nutrient media on *in vitro* micropropagation of *Bacopa monnieri* after 4 weeks of culture initiation

Nutrient media	Shoot Formation		Rooting	
	Number of shoots/explant	Shoot length (cm)	Number of roots/explant	Root length (cm)
Schenk and Hildebrandt (SH)	3.31 bc	2.20 de	5.50 de	2.99 de
SH+MSvit	2.31 cd	5.03 ab	4.50 e	4.20 c
Murashige and Skoog (MS)	1.44 d	**5.94 a**	6.00 cde	2.46 ef
Anderson's Rhod.	**6.31 a**	2.41 de	4.25 e	1.66 f
CHU(N_{6})	2.62 bcd	2.79 cd	8.50 bc	3.10 de
McCown	2.56 bcd	3.18 cd	5.75 de	2.40 ef
Orchimax	4.00 b	2.58 cde	**14.50 a**	**7.15 a**
Lindemann Orchid	2.75 bcd	3.30 cd	6.50 cde	3.27 cde
Knudson C orchid	2.81 bcd	4.02 bc	6.75 cde	3.82 cd
MS No: 3B	5.75 a	1.12 e	9.75 b	3.01 de
MS No: 1B	3.12 bc	2.51 cde	8.00 bcd	3.07 de
SH+B_5 vitamins	3.75 bc	3.63 bcd	8.50 bc	**6.01 b**

The difference between the means shown in different letters in the same column is significant at the 0.05 level according to the Tukey test results. 30 g/l sucrose and 5 g/l agar were added to the nutrient media.

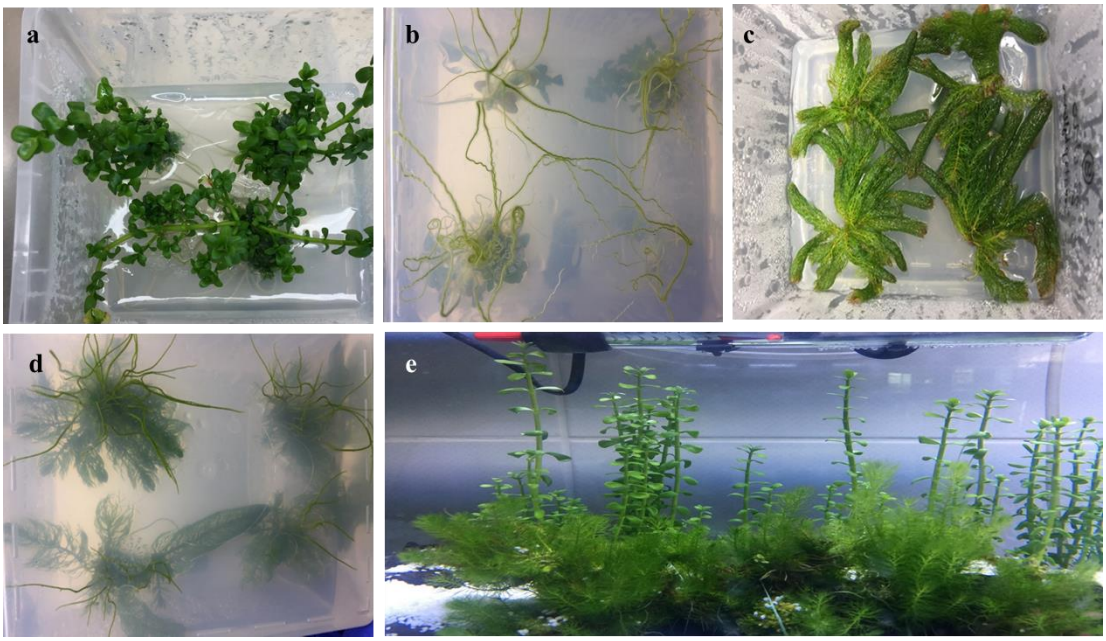

Figure 1. Micropropagation of *B. monnieri* and *C. demersum* 4 weeks after the culture initiation and growth in the aquarium. a) Shoot regeneration in *B. monnieri* on Anderson's Rhododendron medium containing 30 g/l sucrose and 5 g/l agar, b) Root formation in *B. monnieri* in Orchimax medium containing 30 g/l sucrose and 5 g/l agar, c) shoot regeneration in *C. demersum* on Knudson C orchid medium containing 30 g/l sucrose and 5 g/l agar, d) root formation in *C. demersum* in SH medium supplemented with 50 g/l sucrose and 5 g/l agar, e) growth of *B. monnieri* (in the background), and *C. demersum* in aquarium (in the foreground)

Table 2. Effect of different basal nutrient media on *in vitro* micropropagation of *Ceratophyllum demersum* after 4 weeks of culture initiation

Nutrient media	Shoot Formation		Rooting	
	Number of shoots/explant	Shoot length (cm)	Number of roots/explant	Root length (cm)
SH	8.75 b	3.92 cd	11.50 ab	2.12 b
SH+MSvit	7.25 c	3.67 cd	10.25 bc	**2.97 a**
MS	5.75 e	3.66 cd	5.50 e	1.92 bc
Anderson's Rhod.	7.25 c	4.12 bc	2.25 g	0.34 e

CHU(N_6)	3.00 f	2.17 f	3.50 fg	1.02 d
McCown	9.75 ab	4.62 ab	4.25 ef	0.62 de
Orchimax	6.25 cde	3.05 e	**12.25 a**	2.90 a
Lindemann Orchid	6.25 cde	3.07 e	9.00 c	3.08 a
Knudson C orchid	**10.25 a**	**4.80 a**	5.75 de	1.52 c
MS No: 3B	5.75 e	3.50 de	5.25 e	0.90 d
MS No: 1B	7.00 cd	3.85 cd	2.75 fg	0.37 e
SH+B_5 vit	6.00 de	3.57 de	7.25 d	1.87 bc

The difference between the means shown in different letters in the same column is significant at the 0.05 level according to the Tukey test results. 30 g/l sucrose and 5 g/l agar were added to the nutrient media.

Although their content depends on a number of variables such as genotype, explant and purpose of the study, basic nutrient media contain all macro and micro elements and vitamins necessary for plants (Saad and Elshahed 2012). By reducing or increasing these nutritional elements, it is possible to achieve success even in plants whose *in vitro* regeneration is very difficult (Saad and Elshahed 2012; Phillips and Garda 2019). In the present study, the effectiveness of 12 different basic nutrient media developed for different plant species was tested on two different aquarium plant species. Considering the shoot formation and rooting results, it was observed that the basic nutrient media SH for *C. demersum* and Orchimax for *B. monnieri* provided the highest rate of micropropagation. On the other hand, in previous studies with aquarium plants, only one or two basic nutrient media were used at the same time, and the most commonly used was the standard MS nutrient medium (Ceasar et al., 2010; Sharma et al., 2010; Koul et al. 2014; Barpete et al., 2015; Chaunhan and Shirkot 2020, Özcan et al., 2021). Apart from MS nutrient medium, B_5 (Koul et al. 2014) and SH media have also been used, although rarely, in different plant species (Özcan et al. 2023). However, in the present study, standard MS nutrient medium was not effective in both species. This result revealed the necessity of making adjustments in the nutrients contained in the developed basic nutrient media for high success in different plant species.

Effect of different sucrose concentrations

In optimizing the sucrose ratio for *B. monnieri*, Orchimax medium which gave the highest results in the determination of media was used. The effect of sucrose ratios on the number of shoots per explant was found to be statistically insignificant. While the highest number of shoots per explant was obtained from the addition of 20 g/l sucrose with 3.38, the highest shoot length was found in the sucrose-free medium with 6.87 cm (Table 3). While the effect of sucrose ratios on root length in *B. monnieri* was found to be statistically insignificant, the highest number of roots per explant was obtained from 30 g/l with 11.75, and the maximum root length was obtained from 60 g/l sucrose ratio with 2.74 cm (Table 3). Considering shoot

formation and rooting together, it was observed that the majority of sucrose ratios were effective in *B. monnieri*. However, 20 g/l sucrose was used to optimize the agar ratio.

To determine the effect of different sugar ratios on *C. demersum*, SH medium which gave the highest micropropagation results in the nutrient media study was used. The highest number of shoots per explant (6.06) and the highest shoot length (4.93 cm) were accomplished from the SH medium containing 50 g/l sucrose (Table 4). Likewise, the highest number of roots per explant (11.12) and root length (3.45 cm) were also recorded in 50 g/l sucrose application. Additionally, high amounts of sucrose (50-60 g/l) caused reddening of shoot tips. According to these results, the most suitable amount of sucrose for *C. demersum* was 50 g/l (Table 4).

Table 3. Effect of different sucrose concentrations on *in vitro* micropropagation of *B. monnieri* after 4 weeks of culture initiation

Sucrose (g/l)	Shoot Formation		Rooting	
	Number of shoots/explant	Shoot length (cm)	Number of roots/explant	Root length (cm)
0	2.81[nd]	**6.87 a**	10.38 ab	2.32 [nd]
10	2.25	6.56 a	9.62 ab	1.99
20	**3.38**	4.74 ab	8.75 ab	2.31
30	3.00	4.43 ab	**11.75 a**	2.45
40	1.81	5.56 ab	8.31 ab	2.40
50	2.69	2.50 b	5.06 b	2.22
60	2.81	3.11 b	5.75 b	**2.74**

The difference between the means shown in different letters in the same column is significant at the 0.05 level according to the Tukey test results. nd= not significant. Orchimax medium supplemented with 5 g/l agar was used.

Table 4. Effect of different sucrose concentrations on *in vitro* micropropagation of *C. demersum* after 4 weeks of culture initiation

Sucrose (g/l)	Shoot Formation		Rooting	
	Number of shoots/explant	Shoot length (cm)	Number of shoots/explant	Root length (cm)
0	2.31 b	2.10 cd	1.25 b	0.38 c
10	6.12 a	1.99 d	4.31 b	0.98 c
20	5.06 ab	2.42 bcd	3.25 b	2.61 b
30	5.625 ab	3.54 abcd	5.75 ab	2.36 b
40	6.44 a	4.10 ab	5.75 ab	2.93 ab
50	**6.06 a**	**4.93 a**	**11.12 a**	**3.45 a**

60	3.38 ab	3.85 abc	4.38 b	2.74 b

The difference between the means shown in different letters in the same column is significant at the 0.05 level according to the Tukey test results. SH medium supplemented with 5 g/l agar was used.

Although it varies depending on the plant species, the most commonly used sucrose ratio in *in vitro* culture is 30 g/L (Miroshnichenko et al. 2017, Doğan 2020b, Gao et al. 2020). On the other hand, in the present study, the highest micropropagation results were obtained when 20 g/L sucrose was added to the nutrient medium in *B. monnieri* and 50 g/L sucrose in *C. demersum*. Similar results were also reported in *B. monnieri* (Ranjan and Kumar 2018) with the application of 20 g/L sucrose and *R. fluitans* (Özcan et al. 2021) with the addition of 15 g/L sucrose to the nutrient medium.

Effect of Different Agar Concentrations

In the agar application, Orchimax medium containing 20 g/l sucrose were used for *B. monnieri*. The results of the different agar applications on shoot and root formation in *B. monnieri* are given in Table 5. While the highest number of shoots per explant (3.50) was obtained from the medium added with 5 g/l agar, the highest shoot length (6.89 cm) was obtained from the medium solidified with 7 g/l agar. The highest number of roots per explant (8.06) was observed in nutrient medium solidified with 5 g/l agar. The highest root length was found in agar-free medium with 7.26 cm (Table 5). When shoot formation and rooting are considered together, the highest micropropagation results in *B. monnieri* were obtained from the medium solidified with 5 g/l agar.

Table 5. Effect of different agar concentrations on *in vitro* micropropagation of *B. monnieri* after 4 weeks of culture initiation

Agar (g/l)	Shoot Formation		Rooting	
	Number of shoots/explant	Shoot length (cm)	Number of shoots/explant	Root length (cm)
0	2.50 b	3.37 c	4.81 c	**7.26 a**
1	2.31 bc	6.32 ab	4.69 c	4.67 b
2	2.56 ab	2.98 c	5.19 c	3.82 b
3	2.25 bc	3.85 bc	6.38 abc	3.52 b
4	2.81 ab	4.50 abc	7.87 ab	3.27 b
5	**3.50 a**	5.35 abc	**8.06 a**	3.01 b
6	1.37 cd	5.51 abc	6.06 bc	2.91 b
7	1.25 d	**6.89 a**	5.81 c	2.62 b

The difference between the means shown in different letters in the same column is significant at the 0.05 level according to the Tukey test results. Orchimax medium supplemented with 20 g/l sucrose was used.

In the agar experiment for *C. demersum*, SH medium supplemented with 50 g/l sucrose were used. The effect of different agar concentrations on the micropropagation of *C. demersum* is given in Table 6. The highest shoot formation and rooting were achieved from liquid medium without agar. In this medium, the number of shoots per explant was 10.69 and the shoot length was 6.26 cm (Table 6). The lowest shoot formation and rooting were recorded in SH medium solidified with 7 g/l agar. According to these results, the most suitable nutrient medium for *C. demersum* was liquid medium without agar.

C. demersum achieved the highest micropropagation in liquid medium without agar and *B. monnieri* in nutrient medium containing 5 g/L agar. The fact that plants have different *in vitro* responses to different agar ratios suggests that it may be related to their ability to survive under or above water. For example, the fact that *C. demersum* can live completely under water and without roots is an explanation for the fact that it shows its best development in *in vitro* liquid culture without the need for solid nutrient media. Karataş *et al*. (2014) in their study, they obtained the highest rate of *in vitro* micropropagation of *C. demersum* from liquid nutrient media, as in the present study. While *B. monnieri* can grow in water-rich and water-saturated soils (Visnoi et al. 2016), it can also be grown in field conditions (Rahe et al. 2020). This explains why *B. monnieri* requires a more solid medium than *C. demersum* in tissue culture.

Table 6. Effect of different agar concentrations on *in vitro* micropropagation of *C. demersum* after 4 weeks of culture initiation

Agar (g/l)	Shoot Formation		Rooting	
	Number of shoots/explant	Shoot length (cm)	Number of shoots/explant	Root length (cm)
0	**10.69 a**	**6.26 a**	**10.50 a**	**4.30 a**
1	7.19 b	5.84 a	8.00 ab	3.65 ab
2	6.31 b	5.39 a	8.81 ab	3.29 bc
3	6.00 bc	4.02 b	6.50 ab	2.80 bcd
4	6.56 b	4.06 b	9.50 ab	2.30 cd
5	4.88 bcd	3.90 b	6.75 ab	2.87 bcd
6	3.38 d	2.51 c	3.06 b	2.65 bcd
7	3.81 cd	2.00 c	4.12 ab	2.08 d

The difference between the means shown in different letters in the same column is significant at the 0.05 level according to the Tukey test results. SH medium supplemented with 50 g/l sucrose was used.

CONCLUSIONS

With present study, *Ceratophyllum demersum* and *Bacopa monnieri* species, which are important plants in aquariums and have medicinal value and water phytoremediation properties, were able to be propagated at a high rate within 4 weeks. Agar, the most expensive compound of nutrient media, was not required for the propagation of *C. demersum*. Moreover, in this species sucrose used in low amounts gave the highest results. *In vitro* propagated plants were acclimated to aquariums at a 100% rate. The methods developed for the plant species studied have low economic costs and can also be used for commercial production.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest. The opinions and/or conclusions expressed are exclusively those of the authors.

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