ORGAN AND PLANTLET REGENERATION OF *MENYANTHES TRIFOLIATA* THROUGH TISSUE CULTURE

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ABSTRACT

The conditions for the regeneration of plants through organogenesis from callus tissues of *Menyanthes trifoliata* are described. The shoot multiplication rate was affected by basal culture media, the type and concentration of cytokinin and subculture number. The best response was obtained when caulogenic calli were cultured on the modified Schenk and Hildebrandt medium (SH-M) containing indole-3-acetic acid (IAA 0,5 mg/l) and 6-benzyladenine (BA 1 mg/l) or zeatin (2 mg/l). Under these conditions *ca* 7 shoots (mostly 1 cm or more in length) per culture in the 5th and 6th passages could be developed. In older cultures (after 11-12 passages) there was a trend for more numerous but shorter shoot formation. All regenerated shoots could be rooted on the SH-M medium supplemented with 0.5 mg/l IAA within 6 weeks; 80% of in vitro rooted plantlets survived their transfer to soil.

KEY WORDS: *Menyanthes trifoliata*, caulogenic calli, multiplication rate, plantlet regeneration.

INTRODUCTION

Menyanthes trifoliata L. (Menyanthaceae) is a marsh plant used in officinal (Hagers 1976) and traditional medicine (Nowiński 1980) as amarium, tonicum and stomachicum agents. The leaves have also cholagogic and choleretic properties (Kroeber 1937). They are used in pharmaceutical industry as components of many herbal and galenic preparations. Many kinds of pharmacologically active compounds, such as secoiridoids (Junior 1989), flavonoids, phenolic acids (Bohm et al. 1986) and coumarins (Adamczyk et al. 1990) have been found in *M. trifoliata*.

Recently, the number of places where *M. trifoliata* occurrs has decreased as the meadows and peat bogs, which are the natural environment of this plant, are being meliorated. In many regions of Poland *M. trifoliata* has been indiscriminately exploited and has become an endangered species. Micropropagation of *M. trifoliata* through in vitro techniques might be a possibility to overcome this problem and to increase the availability of the plant. The only previous work dealing with *M. trifoliata* tissue culture in vitro was reported by Wysokińska et al. (1985). In that study the authors investigated the production of secoiridoids, coumarins and phenolic acids in callus and suspension cultures of *M. trifoliata*. They also regenerated shoots from callus tissue on B5 (Gamborg et al. 1968) medium supplemented with IAA (2 mg/l) and BA (2 mg/l).

The aim of our study was to develop a procedure for in vitro plant regeneration of *M. trifoliata* through adventitious shoots. The appropriate media for shoot multiplication and rooting were also selected.

MATERIAL AND METHODS

Establishment of culture

Seeds of *M. trifoliata* were obtained from the Botanical Garden in Bonn. Seeds were surface sterilized by immersion in 2.5% sodium hypochlorite for 15 min and were rinsed three times in sterile distilled water. Then they were germinated in aseptic conditions on MS (Murashige and Skoog 1962) medium supplemented with 0.02 mg/l of kinetin, gibberellic acid (GA_3) (50 mg/l) and Difco Bacto Agar (0.8%).

In order to induce the growth of calli, 4-5 -week- old seedlings were used. Explants were prepared as follows: cotyledons were cut in half; hypocotyls and roots were cut into 0.5-1 cm long segments. These explants were cultured on SH (Schenk and Hildebrandt 1972) medium with 0.2 mg/l indole-3-acetic acid (IAA), 0.2 mg/l 1-naphthaleneacetic acid (NAA) and 2.0 mg/l 6-benzyladenine (BA). Some calli formed adventitious buds and shoots on the medium within 8 weeks. The calli from cotyledons were the most suitable for organogenesis. Therefore, they were utilized in subsequent experiments.

Shoot multiplication

For shoot multiplication, the caulogenic calli were cut into pieces (with one bud each) which were cultured on SH modi-

fied (SH-M) medium supplemented with 20 g/l sucrose and 0.8% agar. SH-M medium was based on microelements and vitamins of the Schenk and Hildebrandt (SH) medium. The composition of macroelements in SH-M medium is shown in Table 1. The growth regulators added were 0.5 mg/l IAA and different concentrations of 6-(4-hydroxy-3-methyl-but-2-enylamino) purine (zeatin), 6-(,-dimethylallyloamino) purine (2iP), 6-furfurylaminopurine (kinetin) or BA as indicated in Table 2. The multiplication rate (MR) was evaluted as the mean number of shoots and buds obtained in one culture at the end of each multiplication cycle (6 weeks). The length of regenerated shoots and the percentage of shoots longer than 1 cm were also recorded after 6 weeks.

Culture media

Five different basal media SH, SH-M, B5 (Gamborg et al. 1968), MS and NN (Nitsch and Nitsch 1969) were used to examine their effect on shoot formation and their development. The media were supplemented with IAA (0.5 mg/l) and BA (1 mg/l). The number of shoots per culture (MR), shoot length and percentage of shoots longer than 1 cm were recorded after 6 weeks of growth on these media.

Rooting

To induce rooting, individual shoots (1 to 2 cm long) cultured on SH-M medium with IAA (0.5 mg/l) and BA (1mg/l) for 6 weeks, were excised from the callus and transfered onto SH-M medium without growth regulators. The same basal media containing IAA or NAA at 0.5 and 1 mg/l were also used. Rooting percentage and number of roots per shoot as well as root length were recorded after 3 and 6 weeks.

Culture conditions

In all the experiments described above the pH of the media was adjusted to 5.6-5.8 and 0.8% agar was added before autoclaving. All the media were autoclaved for 15 min at 121°C. The cultures were kept in cool white fluorescent light (40 µmol m^{-2} s^{-1}) at the temperature of 26°C.

All the experiments concerning shoot multiplication and rooting were repeated twice and there were ca 15 replicates per combinations in each set.

Acclimatization of plants

Rooted shoots (6 week old) were washed in sterile water to remove agar from the roots and transferred to pots with a sterilized mixture of soil, sand and peat (3:1:1 v/v). The plantlets were acclimatized for 3-4 weeks at 26°C, in continuous light (cool white fluorescent lamps, 40 µmol m^{-2} s^{-1}). Glass covers were used to ensure high humidity around the plants at the initial stages of growth. After 14 days the glass covers were removed.

TABLE 1. Modified SH medium (SH-M) used for micropropagation of *M. trifoliata*.

Constituent*	Concentration (mg/l)
NH_4NO_3	400
$Ca(NO_3)_2 \cdot H_2O$	1500
KNO_3	2000
KH_2PO_4	200
KI	0.1
$MgSO_4$ $7H_2O$	400
Sucrose	20000

*Other constituent (microelements and vitamins) as in SH medium (Schenk and Hildebrandt 1972)

RESULTS AND DISCUSSION

The exposure of *M. trifoliata* seedling tissues (cotyledon, hypocotyl and roots) to SH agar medium with 0.2 mg/l IAA, 0.2 mg/l NAA and 2 mg/l BA led to the formation of callus. The callus was yellow-green and formed initially at the site of excision. Gradually the callus covered the whole surface of explants. In some cases, adventitious buds were developed from cotyledon-derived calli, within 8 weeks. The caulogenic calli were transferred onto modified SH media (see Table 1) with 0.2 mg/l IAA, 0.2 mg/l NAA and 2 mg/l BA. After 5 subcultures (6 weeks each), the caulogenic calli were designed to find suitable conditions to induce multiple shoots of *M. trifoliata*.

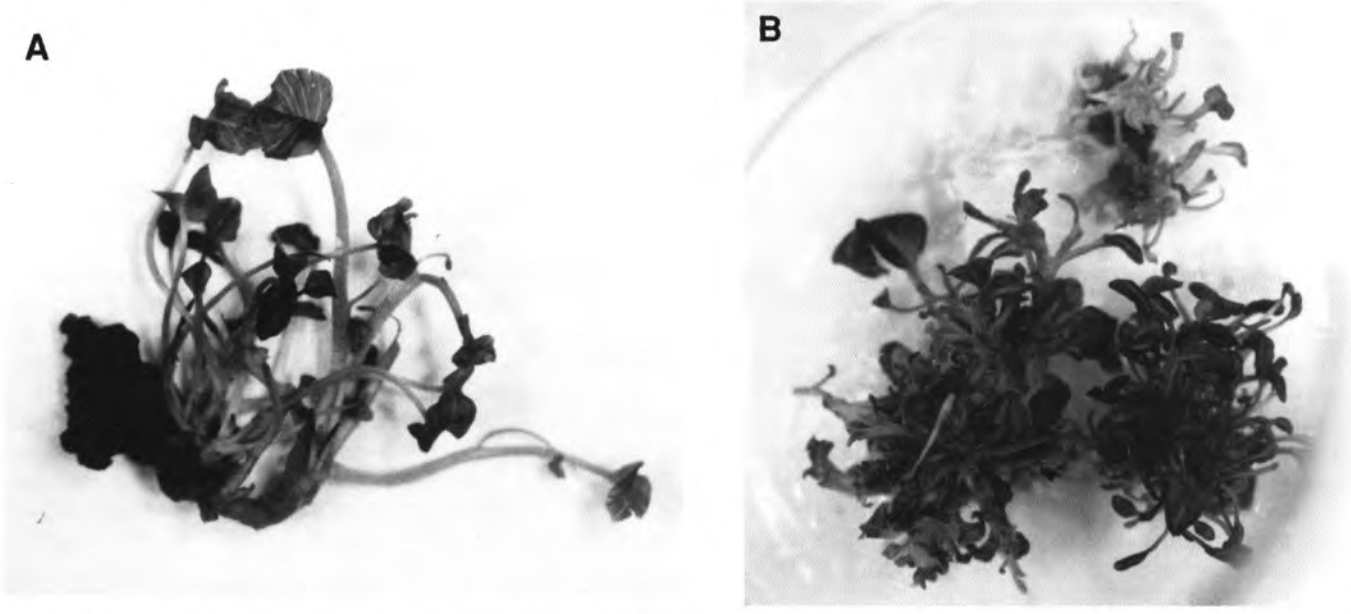

Fig. 1. Shoot formation from callus culture of *M. trifoliata* on SH-M medium containing IAA (0.5 mg/l) and BA (1 mg/l) after 5 subcultures (A) and after 12 subcultures (B) (each subculture lasted 6 weeks).

TABLE 2. The effect of different cytokinins in combination with IAA (0.5 mg/l) on shoot multiplication from callus cultures of *M. trifoliata*.

Cytokinin (mg/l)	% cultures with shoots	Multiplication rate	Mean shoot length [cm] [% shoot ≥ 1 cm]	% cultures with roots
0	90	1.1	1.1 [60]	77.0
ZEATIN				
0.01	90	1.3	1.8 [80.5]	77.5
0.1	90	2.6	1.5 [66.2]	63.8
1.0	100	5.9	1.7 [90.0]	15.0
2.0	100	7.0	2.3 [95.0]	0.0
2iP				
0.01	80	1.1	0.9 [22.2]	37.5
0.1	50	1.8	1.9 [100.0]	60.0
1.0	70	4.3	2.3 [85.0]	38.0
2.0	95	4.0	1.8 [80.0]	35.0
KINETIN				
0.1	30	1.0	0.9 [33.3]	0.0
1.0	65	3.5	0.8 [80.0]	38.0
2.0	80	1.9	0.6 [47.1]	0.0
5.0	80	2.8	0.7 [45.5]	0.0
BA				
0.1	100	2.6	0.9 [79.2]	35.0
1.0	90	7.2	1.5 [90.0]	0
2.0	90	7.3	1.2 [73.2]	0
5.0	80	2.5	0.9 [20.1]	0

Culture conditions: SH-M agar medium.
Growth period 6 weeks; passages 5 and 6.

Shoot multiplication

Different cytokinins (zeatin, 2iP, kinetin and BA) with IAA (0.5 mg/l) were tested for their effects on shoot multiplication. The results (the percentage of cultures showing shooting response, shoot multilication rate, and the length of shoots) are shown in Table 2. The best response was achieved on SH-M medium with IAA and zeatin at the concentration of 2 mg/l. The number of shoots per culture was 7. Almost all regenerated shoots (95%) achieved the length of 1 cm (or more) and they were ready for rooting at the end of each subculture. Multipliation rate as well as shoot quality on media supplemented with 1 mg/l BA (Fig. 1A) can be compared to those with 2 mg/l zeatin (Table 2). The number of shoots per culture was also high (*ca* 7) at the concentration of 2 mg/l BA, but induced shoots were smaller with more or less vitrified appearance. It was therefore, necessary to use a lower concentration of BA (1 mg/l). Other tested cytokinins (2iP and kinetin) were found to be less effective for shoot differentiation in case of *M. trifoliata* (Table 2). For example, on the medium with 1 mg/l 2iP 85% of shoots were 1 cm or longer, but the number of shoots per culture was low (Table 2). Kinetin decreased both, the frequency of shoot formation and the number of induced shoots (Table 2).

Spontaneous root formation was also observed on calli cultured on SH-M media with IAA (0.5 mg/l) and with low cytokinin concentration (Table 2). Some of these roots had direct vascular connection with the shoots and the root-shoot units could be sited in the soil. In most cases, however, the roots developed from calli, independently of the shoots. Such units failed to take root in the soil. It was, therefore necessary to isolate the shoots and root them individually in vitro.

Although usually the ability to produce multiple shoots is gradually lost during the culture period and is generally not maintained for over a year, in *M. trifoliata* it is possible to grow shoot cultures for at least 12 subcultures (1.5 years) with a multiplication rate of 17.5 shoots per culture (SH-M medium suplemented with 0.5 mg/l IAA and 1 mg/l BA) (Fig. 1B; Table 3). It is an improvement over that of 7.2 shoots as reported for culture in the 5-6th passage (Fig. 1A; Table 2). However, it is concomitant with the reduction of shoot length. In the 12th passage only 50% of the produced shoots exceed 1 cm after 6 weeks of culture (Table 3). This contrasted with 90% for cultures which were subcultured five or six times (Table 2). The small shoots (shorter than 1 cm) do not root well and consequently the number of shoots which could be taken at the end of the culture cycle was not considerably affected.

For shoot multiplication of *M. trifoliata*, five basal media SH, SH-M, MS, B5, and NN were used (Table 3). Each of the media tested was supplemented with IAA (0.5 mg/l) and BA (1 mg/l) and solidified with 0.8% agar. It was found that high salt MS medium was better for bud initiation, as compared to the intermediate salt B5 and SH/SH-M media or low salt NN medium (Table 3). However, the shoots induced on the MS medium appeared to be vitrified (partially fasciated, swollen, difficult to harvest). Their growth was inhibited: only 38% of the shoots were 1 cm in length. As suggested by Kevers et al. (1984) and Gaspar et al. (1987) the induction of vitrification can be attributed to high NH_4^+ and Cl^- content in MS medium. However, shoot-buds of *M. trifoliata* initiated on cultures growing on NN medium also grew in clusters and remained short and thick. The medium has a lower concentration of nutrient, especially nitrogen. Among the tested media, SH and SH-M promoted growth of the induced shoot of *M. trifoliata*. The results achieved on SH medium were almost equal to those obtained on SH-M medium, although these

TABLE 3. The effect of different media on *M. trifoliata* shoot formation.

Basal media	% cultures with multiple shoots	Multiplication rate	Mean length [cm] of shoots [% shoot ≥ 1 cm]
SH	85.3	19.0	1.7 [51.0]
SH-M	96.7	17.5	1.4 [50.7]
B5	78.8	11.7	1.1 [45.1]
MS	85.3	26.6	1.1 [38.2]
NN	80.6	17.3	0.8 [21.5]

SH – Schenk and Hildebrandt 1972,
SH-M. – Schenk and Hildebrandt modified medium (Table 1),
B5 – Gamborg et al. 1968,
MS – Murashige and Skoog 1962,
NN – Nitsch and Nitsch 1969.
Culture conditions: agar (0.8%) medium with 0.5 mg/l IAA and 1.0 mg/l BA. Growth period: 6 weeks; passages 11 and 12.

media had different mineral composition (Table 1, see also Schenk and Hildebrandt 1972). Our results suggest that besides mineral composition of the medium, other factors such as subculture number and type and concentration of cytokinin are also of major importance to the production of high quality shoots in *M. trifoliata* cultures.

Rooting and acclimatization

When the shoots grew to 1 cm (or more) in height, they were cut off and transferred onto a fresh medium for rooting. Ninety percent of shoots rooted on SH-M medium without growth regulators. The roots were long, streight and the formation of lateral roots was almost completely inhibited (Fig. 3). The roots appeared after 3 weeks, but the shoots could be acclimatized after 6 weeks in order to increase the number of roots per shoot and to obtain sufficient shoot elongation. The results presented here showed that in *M. trifoliata* as in most other species, auxin increased the number of roots per rooted shoots, but decreased the length of formed roots. The effects of IAA and NAA (at the concentrations of 0.5 and 1 mg/l) on basic rooting parameters are presented in Figs 2 and 3.

The highest rooting efficiency was obtained with 0.5 mg/l IAA. Roots induced by IAA were long (up to 3 cm), thin and developed directly from the cut end of the shoot without intermediate callus (Fig. 3). The effect of NAA on rooting is different from that of IAA. NAA leads to the formation of numerous very short and thick roots (Fig.3). Furthermore, the rooting was inhibited by NAA (72% and 30% shoots formed roots in the presence of 0.5 mg/l and 1 mg/l NAA, respectively) (Fig. 1). The inhibition of root formation and their elongation combined with small callus formation at the shoot base (especially at the concentration of 1 mg/l) eliminated any positive effect of NAA on the number of roots. The callogenic effect of NAA had already been reported by Alderson et al. (1987).

The rooted shoots (2 cm or longer) were transferred onto a soil mixture and they were growing in continuous light and in high air humidity to prevent desiccation and to stimulate their growth. Acclimatization on a small scale in the culture chamber for 3 to 4 months gave on 80% survival rate (Fig. 4). Growth in field conditions remains to be studied.

In this study we have successfully induced the organogenesis in callus culture of *M. trifoliata* and obtained the regeneration of complete plants. Normal shoot growth and subsequent plantlet regeneration could be achieved on both SH or SH modified agar media supplemented with IAA (0.5 mg/l) and BA (1 mg/l) or zeatin (2 mg/l). Separate shoot elongation stage was not necessary. Shoot proliferation and development on the same culture medium would facilitate the micropropagation procedure, by reducing the three stages (bud induction, shoot growth and rooting) to two (induction/growth and rooting). This could be beneficial by reducing the production costs and shortening the time demanded for the complete plantlet regeneration.

There is an increasing interest in *M. trifoliata* as a medicinal plant. Its in vitro cultures can become a promising method for the conservation of this endangered plant species and pro-

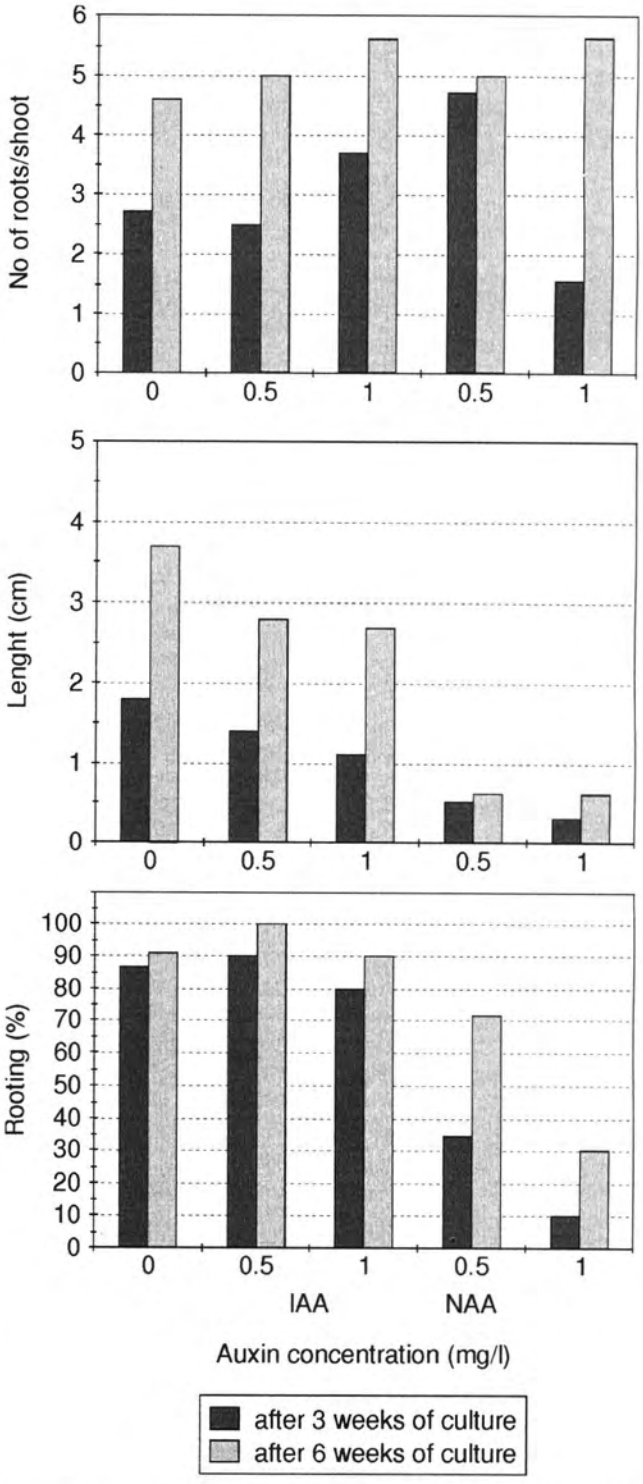

Fig. 2. Rooting response of shoots of *M. trifoliata* to auxin on SH-M medium after 3 and 6 weeks.

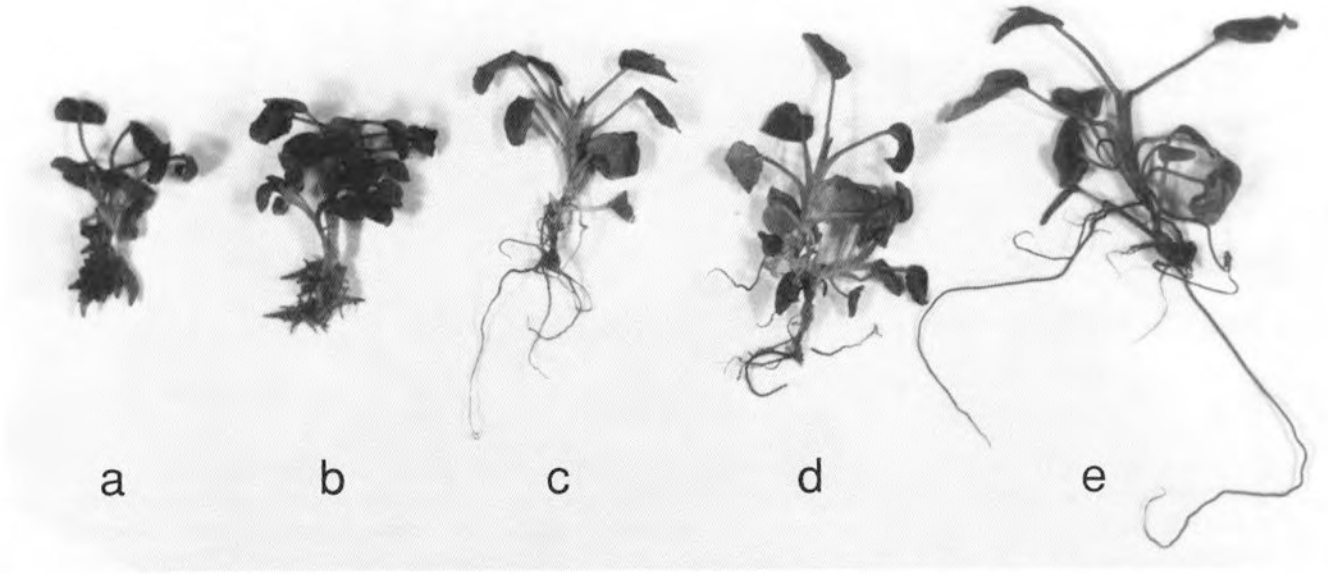

Fig. 3. Comparison of the development of roots formed on SH-M medium with (a) NAA 0.5 mg/l; (b) NAA 1 mg/l; (c) IAA 0.5 mg/l; (d) IAA (1 mg/l) and (e) without growth regulators. Culture period: 6 weeks.

Fig. 4. *M. trifoliata* plants derived from micropropagation, after 3 months of acclimatization.

vide plant material for phytochemical analyses. In the future plant regeneration described here may promote the possibility to obtain transgenic plants of *M. trifoliata*. Such plants might play an important role in the improvement of disease resistance and perhaps also enhance the production of secondary metabolites.

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REGENERACJA ORGANÓW I ROŚLIN *MENYANTHES TRIFOLIATA* W KULTURACH TKANKOWYCH

STRESZCZENIE

Opisano warunki dla organogenezy i regeneracji roślin *Menyanthes trifoliata* w hodowli in vitro. Intensywność mnożenia pędów z tkanki kalusowej *M. trifoliata* zależy od zastosowanego podłoża, rodzaju i stężenia cytokininy oraz od wieku kalusa. Najlepsze rezultaty osiągnięto hodując kaulogenną tkankę kalusową na zmodyfikowanym podłożu Schenka i Hildebrandta (SH-M) uzupełnionym IAA w ilości 0,5 mg/l i BA w ilości 1 mg/l lub zeatyną w ilości 2 mg/l. W tych warunkach w pasażu 5 i 6 uzyskano ok. 7 pędów na kulturę. W 11 i 12 pasażu namnożonych pędów było znacznie więcej (ok. 14/kalus), ale były one krótsze (ok. 50% 1 cm). Na podłożu SH-M z IAA 0,5 mg/l po 6 tygodniach ukorzeniło się 100% pędów. Z tych roślin około 80% przeżyło po przeniesieniu do gleby.

SŁOWA KLUCZOWE: *Menyanthes trifoliata*, kaulogenny kalus, współczynnik mnożenia pędów, regeneracja roślin.