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EFFICIENT *IN VITRO* REGENERATION OF MEDICINAL AQUATIC PLANT WATER HYSSOP (*Bacopa monnieri* L. PENNELL)

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Micropropagation of medicinal plants under *in vitro* conditions provides an alternative for the isolation of medicinally active compounds by applying biotechnological tools. The present study reports an efficient and repeatable protocol of adventitious shoot regeneration of water hyssop (*Bacopa monnieri* L. Pennel) a semi aquatic plant by culturing leaf explants on MS medium supplemented with various concentrations of BA and TDZ. Leaf explants responded variably to both growth regulators. Direct and indirect adventitious shoot regeneration on leaf explant was recorded on MS medium containing various concentrations of BA. Whereas, TDZ containing medium initiated shoot buds and shoot induction after the explants were transferred to MS medium. Maximum number of 26.67 and 28.25 shoots per explant was recorded on MS medium containing 2.0 mg l^{-1} BA and 0.2 mg l^{-1} TDZ respectively. In general, longer shoots were recorded on BA containing medium compared to TDZ containing medium. However, mean shoot length decreased with each increase in the concentration of both plant growth regulators in the culture medium. Regenerated shoots were successfully rooted on IBA containing MS medium and were acclimatized successfully in pots containing organic matter or jars containing water. Establishment of reliable, successful multiplication protocol of *B. monnieri* using biotechnological tools is an important step for multiple use of the plant as ornamental plant, natural water cleaner for safe environment and rapid extraction of medicinally important compounds from this important aquatic plant.
Keywords: Adventitous shoots, regeneration, aquatic plant, mass proliferation

INTRODUCTION

Medicinal plants are being used since ancient times for curing of diseases and are still in use as traditional medicine or as a source of medicinally important compounds. Water hyssop *(Bacopa monnieri* L. Pennel) has great importance in traditional medicine system due to active compounds like alkaloids, saponins, flavonoids, betulic acid, stigmastrol, beta-sitosterol and bacopasaponins (Ali *et al*., 1999; Chatterji *et al*., 1963, 1965). It is also used as a cardiac tonic, brain tonic to enhance memory development, and to provide relief to patients with anxiety or epileptic disorders in traditional medicinal systems of Pakistan and India (Chopra, 1958; Mukherjee and Dey, 1996; Vijaykumar *et al*., 2010). It possesses anti-inflamatory, analgesic, antipyretic and diuretic activity (Vohora *et al*., 1997; Stough *et al*., 2001). It is also used to treat insanity, epilepsy, hoarseness, enlargement of spleen, snake bite, rheumatism, leprosy, eczema, ring worm (Basu and Walia, 1994) and treat anxiety, epilepsy, bronchitis, asthma, irritable bowel syndrome and gastric ulcers (Shakoor *et al*., 1994).

The plant is perennial creeping herb with relatively thick succulent leaves that are oblanceolate that are arranged oppositely on the stems. It commonly grows in damp and marshy places throughout South Asia up to an altitude of 1320 m. The plant bears small, white coloured flowers with four or five petals. Water hyssop is very popular aquarium plant in Turkey due to its appearance and adaptability under slightly brackish conditions.

Light-emitting diode (LED) is a semiconductor light source (Anonymous, 2005); which is more energy-efficient, emit less heat and can provide optimum light frequency for plant growth and blooms period. It can provide an alternate of fluorescent lamps during *in vitro* shoot regeneration (Lian *et al*., 2002; Li *et al*., 2010). Li *et al*. (2010) reported larger, healthier plantlets and a greater biomass of upland cotton in the presence of red LED supplemented with a quantity of blue LED light. They also reported blue and red LED (B:R = 1:1) as most suitable light for the growth of upland cotton plantlets *in vitro*.

Previously, Sharma *et al*. (2010) and Vijaykumar *et al*. (2010) have reported *in vitro* propagation of *B. monnieri.* However, there is still need to develop more reliable protocols for this highly important medicinal, rock gardens and ornamental aquarium plant using different explants. There is also need to develop acclimatization protocol of tissue cultured plants under aquatic conditions due to its importance as aquatic ornamental plant. The present study aimed *in vitro* adventitious shoot regeneration, rooting and acclimatization of the plant both on soil and in water using red:blue LED lights.

MATERIALS AND METHODS

The water hyssops plants were obtained from local traders of aquatic plants at Karaman province of Turkey. Plant twigs with 4-5 nodes with attached leaves were washed under tap water for 5 minutes followed by surface sterilizeion with 40% diluted H_2O_2 (v/v) for 10 min. Thereafter, they were rinsed thrice with sterilized bidistilled water by continuous stirring for 5 minutes. The twigs were cultured on MS medium supplemented with 30 g sucrose per litre and solidified with 0.65% agar, devoid of plant growth regulators for 2 weeks to obtain contamination free explants. Thereafter, leaf explants (3-4 mm wide and 6-8 mm long) were excised aseptically from twigs and cultured on MS medium containing 0.25, 0.50, 1.0, 1.50 and 2.0 mg/l BA (Table 1) and 0.10, 0.20, 0.40, 0.80 and 1.60 mg/l TDZ (Table 2). Each of which was supplemented with 3% sucrose solidified with 0.65% agar in Magenta GA7 vessels. The experiments were run in triplicate with the pH of all media adjusted to 5.8 before autoclaving (118 kPa atmospheric pressure, 120°C for 21 min). All cultures were incubated under 16 h light photoperiod (4000 lux) using Red:Blue (4:1) LED (Light Emitting Diodes) lights. To overcome the negative effects of TDZ, the regenerating explants on TDZ containing medium were transferred to MS medium after 4 weeks. The data for both experiments (BA and TDZ) were recorded twice after 4 weeks and 8 weeks.

The regenerated shoots were transferred on agar-solidified MS rooting medium containing 0.25, 0.50 and 1.0 mg/l IBA in Magenta GA7 vessels for rooting. Well developed, healthy plantlets were acclimatized after 2 weeks by removing gel adhering to roots of the micropropagated plantlets and acclimatized in pots containing organic matter or were transferred directly to jars containing water. The pots were covered with polythene bags for 1 week and then left open for acclimatization in growth room. Both pots and jars were kept under red:blue (4:1) LED lights under temperature range of 26 ± 2°C.

Each treatment contained 8 explants and was replicated 6 times (8 x 6 = 48 explants) in both shoot and root regeneration experiments and were repeated twice. Statistical analysis was performed as One Way ANOVA using SPSS17 for Windows and post hoc tests were performed using DMRT test. Data given in percentages were subjected to arcsine transformation before statistical analysis.

RESULTS

The study presents the efficient adventitious shoot regeneration from leaf explant of medicinal aquatic plant (water hyssop) cultured on MS medium containing various

Table 1. Effect of various concentrations of BA on adventitious shoot regeneration of *B. monnieri*

BA (mg/l)	Induction of thick mass of cells (%)	Frequency of shoot regeneration (%)	Number of shoots per explant		Change in shoots per explant (%)	Mean shoot length (cm)		Change in mean shoot length (%)
			After 4 weeks of culture	After 8 weeks of culture		After 4 weeks of culture	After 8 weeks of culture	
0.25	100^{ns}	100^{ns}	9.50c	18.33c	92.95c	1.82a	2.45a	34.62c
0.5	100	100	11.38bc	22.67b	99.21a	1.82a	2.33a	28.02d
1.0	100	100	12.04b	23.33b	93.77b	1.29ab	1.83b	41.86a
1.5	100	100	12.46b	23.67b	89.97d	1.23b	1.67b	35.77b
2.0	100	100	15.83a	26.67a	68.48e	0.98b	1.33b	35.71b

Means followed by different small letters within columns are significantly different using DMR test at P<0.005

Table 2. Effect of various concentrations of TDZ on adventitious shoot regeneration of *B. monneri*

TDZ (mg/l)	Induction of thick mass of cells (%)	Frequency of shoot regeneration (%)		Number of shoots per explant		Change in shoots per explant (%)	Mean shoot length (cm)		Change in mean shoot length (%)
		After 4 weeks of culture	After 8 weeks of culture	After 4 weeks of culture	After 8 weeks of culture		After 4 weeks of culture	After 8 weeks of culture	
0.10	100^{ns}	16.67d	100ns	1.00c	16.63c	1563a	0.70a	1.54a	120e
0.20	100	66.67a	100	5.00a	28.25a	465d	0.55ab	1.41b	156a
0.40	100	66.67a	100	4.50a	24.79b	451d	0.55ab	1.32c	140b
0.80	100	50.00b	100	2.33b	18.96c	714c	0.50b	1.17d	134bc
1.60	100	33.33c	100	1.00c	11.67d	1067b	0.45b	1.01e	124cd

Means followed by different small letters within columns are significantly different using DMR test at P<0.005

concentrations of BA and TDZ followed by rooting and acclimatization.

Adventitious shoot regeneration on MS medium containing BA**:** The leaf explants were very prone to shoot regeneration on MS medium containing different concentrations of BA. Direct adventitious shoot regeneration started earlier within 7-8 days of culture on leaf explant with the induction of shoot initials (Fig. 1a). Shoot regeneration (Table 1) was recorded on all explants irrespective of BA concentration in the culture medium. No difference in the ontogenetic behavior of the developing shoots was recorded in terms of shoot regeneration on the data recorded after 4 and eight weeks of culture. Number of shoots per explant increased with increase in BA concentration and ranged 9.50-15.83 shoots with maximum shoots per explant on MS medium containing 2.0 mg/l BA after 4 weeks of culture (Fig. 1b).

Number of shoots per explant after four and eight weeks of culture showed almost parallel behavior. An analysis of results after eight weeks of culture showed an increase in the number of shoots per explant (Fig. 1c) that ranged 18.33-26.67 with maximum shoots on MS medium supplemented with 2.0 mg/l BA. The change (%) in shoots per explant ranged 68.48-99.21%. Mean shoot length decreased with increase in BA concentration. It ranged 0.98-1.82 cm and 1.33-2.45 cm after 4 and 8 weeks of culture respectively (Table 1). Percentage increase in mean shoot length after four and eight weeks of culture ranged 34.62-41.86%.

Adventitious shoot regeneration on MS medium containing TDZ**:** TDZ is a potent urea based cytokinin, which induced shoot buds on leaf explants after 10-12 days of culture (Fig. 2a) and remarkably became thick with growth of lignified walls (Table 2) on all explants on all concentrations of TDZ in the MS medium. The shoot buds conversion into shoots was relatively slow and depressed with small leaves. Shoot initiation started to show only after 3 week of culture. Shoot regeneration frequency of 16.67-66.67 % was recorded after six weeks of culture. Maximum frequency of shoot regeneration was recorded on MS medium containing 0.20 and 0.40 mg/l TDZ. Number of shoots per explant ranged 1.0-5.0 after 4 weeks of culture where both minimum and maximum concentration of TDZ showed inhibitory effects on shoot formation. Maximum number of (5.0) shoots per explant were recorded on MS medium supplemented with 0.20 mg/l TDZ.

The results further indicated that transfer of explants to MS medium after six weeks had positive effect on and it recovered the inhibition caused by TDZ (Fig. 2b). The results indicate 100 % induction of thick mass of cells and shoot regeneration after 8 weeks of culture (Table 2). Transfer to MS medium promoted shoot proliferation and the stunted shoots began to convert into visible shoots after 8th week of culture (Fig. 2c). These ranged 16.63-28.25 shoots per explant after 8 weeks of culture (Fig. 2d). Maximum number of (28.25) shoots per explant were

Figure 1. Adventitious shoot regeneration from leaf explant (a) adventitious shoot regeneration and induction of thick mass of cells after 2 weeks (b) multiple shoot regeneration after 4 weeks and (c) 8 weeks on MS medium containing BA

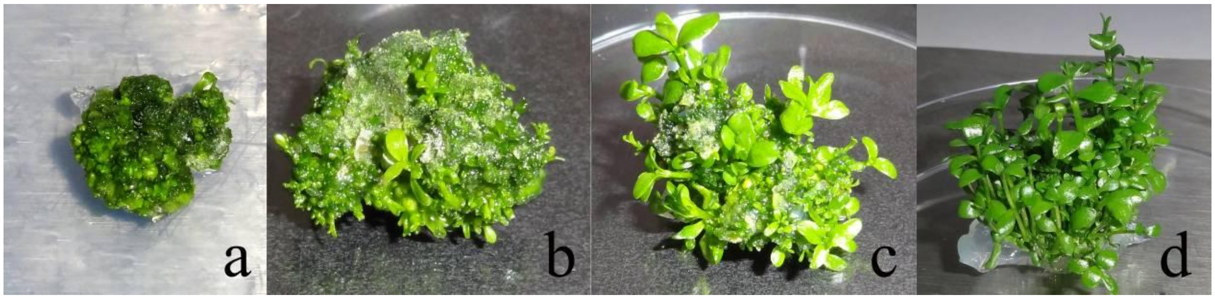

Figure 2. Adventitious shoot regeneration from leaf explant (a) induction of thick mass of cells and shoot buds after 4 weeks on TDZ containing medium (b) shoot initiation after 1 week (c) 2 weeks and, (d) 4 weeks after transfer on MS medium

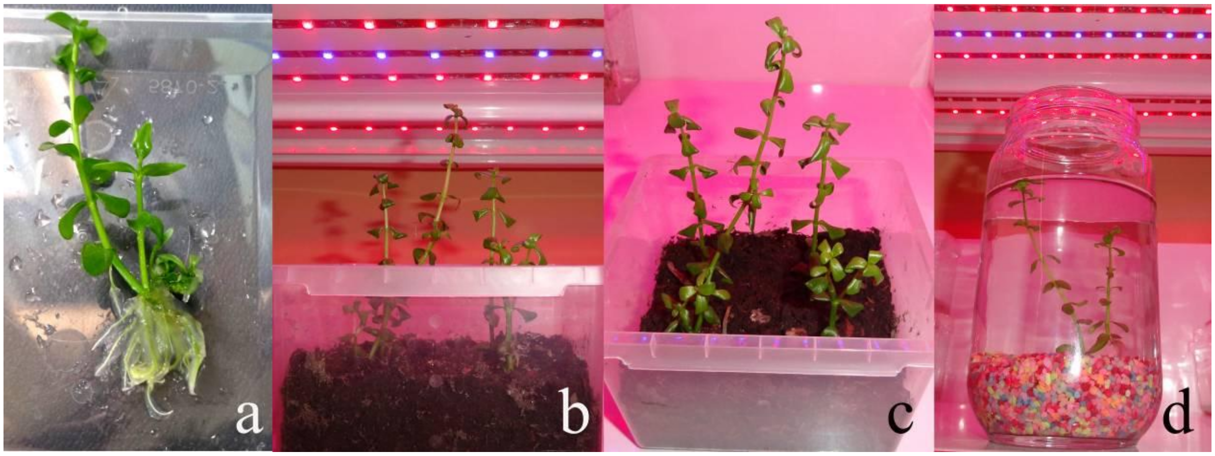

Figure 3. Rooting and acclimatization of *B. monnieri* under LED lights (Red:blue; 4:1). (a) rooted plantlet (b, c) acclimatization in pots containing organic matter (d) acclimatization in water

recorded on MS medium supplemented with 0.20 mg/l TDZ. Comparing the number of shoots per explant after 4 and 8 weeks of culture, the shoot regeneration behavior was not similar. The results further showed that TDZ concentration higher than 0.20 mg/l had partial inhibitory effects on mean number of shoots per explant; which decrease sharply with each increase in the concentration of TDZ. The percentage change in shoot proliferation after transfer to MS medium ranged from 451-1563 % (Table 2). Late shoot induction on TDZ containing medium also resulted in stunning shoots and mean shoot length ranged 0.45-0.70 cm after 4 weeks of culture; which increased significantly after culturing on MS medium and ranged 1.01-1.54 cm after 8 weeks of culture. The results also showed that mean shoot length gradually decreased with each increase in TDZ concentration in the culture medium after 4 and 8 weeks of culture. The positive change of subculturing on mean shoot length was recorded in the range of 120-156 % (Table 2).

***Rooting and acclimatization*: ** Well developed *in vitro* regenerated shoots above 1.0 cm in length from both culture medium were isolated and cultured on MS medium supplemented with variable concentrations of IBA. There was no sign of stress of growth regulators (BA and TDZ) on rooting and root initials started within 3-6 days and 100% rooting was recorded after 2 weeks of culture. However, root initiaion was faster at low concentration of IBA and gradually reduced with increase of IBA concentration in the culture medium. The rooted plantlets also significantly increased in their size in the rooting medium. After 2 weeks of culture, *in vitro* rooted plantlets (Fig. 3a) were acclimatized in pots (Fig. 3b,c) containing organic matter and in jars containing water at pH 7 (Fig. 3d) with continuous circulation of water. In both experiments, plants did not show signs of suppression and acclimatized well and continued their growth. However, rooting and plant growth was relatively fast in liquid culture compared to pots containing organic matter.

DISCUSSION

The present study presents the efficient and reliable protocol for adventitious shoot regeneration of an important medicianal plant water hyssop (*B. monnieri)* using leaf explants. *In vitro* micropropagation of medicinal plants is very important since it is the first step towards isolation of secondary metabolites through tissue culture techniques.

The results showed that leaf explant behaved variably to different concentrations of TDZ and BA in the culture medium. Shoot induction was relatively faster on BA containing MS medium compared to TDZ containing MS medium. However, 100% shoot induction showed the response of leaf explant to both growth regulators and also showed the positive effects of LED lights on shoot regeneration. Vijaykumar *et al*. (2010) reported 30-95% and 50-95% shoot regeneration frequency of *B. monnieri* cultured on BA and TDZ respectively under fluorescent light. The late initiation of shoots by TDZ might be due to suppressive effects of TDZ as has been previously reported in *Cercis canadensis* L. var. alba (Rehder) Bean (Yusnita *et al*., 1990), *Hibiscus rosa-sinensis* L. (Preece *et al*., 1987) and muscadine grape (Gray and Benton, 1991). Higher TDZ concentrations reduced shoot regeneration and resulted in stunted shoots as has been reported for pea (Malik and Saxena, 1992). Similarly, Khawar *et al*. (2004) obtained the highest shoot regeneration from nodal and basal regions of primary shoots developed from seed cultures of lentil on media supplemented with relatively low concentrations of TDZ.

The results further showed that both growth regultors (BA and TDZ) exerted variable response on mean number of shoots per explant. Mean number of shoots increased with increase in BA concentrations. Higher concentrations of BA in the culture medium promoted earlier shoot regeneration and more number of shoot buds compared to lower concentrations of the growth regulator concentrations. The results are contradictory to the findings of Sharma *et al*. (2010) who reported relatively low amount of BA for maximum number of shoots per explants.

Lower concentrations of TDZ in the culture medium was found optimum for maximum shoot regeneration with maximum conversion rate of shoot buds into visible shoots after culturing explants on MS medium. The results showed that suppressive effects of TDZ gradually decreased with time after transfer to MS medium. The results are in line with the findings of Tiwari *et al*. (2001) and Benerjee and Shrivastava (2008) who reported positive effects of subculturing on number of shoots per explants in *B. monnieri.*

On the other hand, increased concentration of both growth regulators in the culture medium showed inhibitory effects on mean shoot length. The results are contradictory to the findings of Vijaykumar *et al*. (2010) who reported increase in shoot length with increase in BA and TDZ concentration in the culture medium using leaf explant of *B. monnieri*. However, relatively longer shoots were recorded from BA containing medium compared to TDZ containing medium supported the findings of Vijaykumar *et al*. (2010). This might be due to early shoot initiation on BA compared to TDZ containing medium. Similarly, Sharma *et al*. (2010) also obtained longer shoots on BA cultured medium compared to Kinetin. However, the conversion rate was relatively high on TDZ containing medium which might be due to subculturing on MS medium which in turn reduced the suppressive effects of TDZ and ultimately resulted in more change in shoot length.

The results on rooting showed the greater response of plants to various concentrations of IBA. Early root initials at low concentration of IBA showed that plants needs relatively less amount of IBA for rooting. Sharma *et al*. (2010) also used IBA for successful rooting in *B. monnieri*. The acclimatization experiments showed establishment of in vitro grown plants both on soil substrate and water. Successful acclimatization in soil has been reported by Sharma *et al*. (2010). However, Karatas *et al.* (2013a) reported successful acclimatization of *in vitro* regenerated plants in water directly. Recently, successful acclimatization of aquatic plants like dwarf hygro (Karatas *et al*., 2013b; Çınar *et al.*, 2013), coontail (Karatass *et al*., 2014a) and Roundleaf toothcup (Karatas et al. 2014b) has been reported.

The study meets objectives of the research. The establishment of successful regeneration, rooting and acclimatization protocol of *B. monnieri* under *in vitro* condition is an important step for application of biotechnological tools to mutiply the plant for multiple use as ornamental plant, for use in water systems to proevent water pollution for provision of safe environment. The protocol can also be applied as base for extraction of medicinally important compounds from this important aquatic plant.

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