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ADVENTITIOUS SHOOT REGENERATION OF THE MEDICINAL AQUATIC PLANT WATER HYSSOP (*BACOPA MONNIERI* L. PENNELL) USING DIFFERENT INTERNODES

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Abstract - Water hyssop *(Bacopa monnieri* L.) is an important medicinal plant due to its active compounds. The plant is also used in ornamental aquaria mainly due to its appearance and adaptability. This study reports on the adventitious shoot regeneration of water hyssop by culturing different internodes and leaf explants on MS media supplemented with various combinations of BA and NAA. All explants induced calli and shoots on all combinations of BA+NAA. The maximum number of shoots per explant on all explants was observed on MS medium supplemented with 0.25 mg/l BA+0.25 mg/l NAA. A higher concentration of NAA inhibited shoot regeneration with all concentrations of BA. Shoots obtained from leaf explants were longer than those from other explants. Regenerated shoots were successfully rooted on MS medium supplemented with IBA. Rooted plantlets were successfully acclimatized in water of various pH levels between 4.0-10.00. It was found that plants can be established on slightly acidic to slightly alkaline media. However, pH 8.0 was found to be more suitable for plant growth under aquatic conditions.

Key words: Adventitious shoot regeneration, aquatic plant, medicinal plant, mass proliferation

INTRODUCTION

Water hyssop *(Bacopa monnieri* L.) is an important traditional medicine plant due to active compounds such as alkaloids (brahmin and herpestine), saponins (d-mannitol and hersaponin, acid A, and monnierin), flavonoids (luteolin and apigenin), betulinic acid, stigmasterol, beta-sitosterol and bacopasaponins (Ali et al., 1999; Chatterji et al., 1963, 1965). In addition, it contains other minor components such as bacopasaponin F, bacopasaponin E, bacopaside N1, bacopaside III, bacopaside IV and bacopaside V (Anbarsi et al., 2006). The plant has been used in traditional medicine in Pakistan and India as cardiac and brain tonic to enhance memory development, and to provide relief to patients with anxiety or epileptic disorders (Chopra, 1958; Mukherjee and Dey, 1996; Vijaykumar et al., 2010). It also possesses anti-inflammatory, analgesic, antipyretic and diuretic activity (Vohora et al., 1997; Stough et al., 2001) and is used to treat insanity, epilepsy, hoarseness, enlargement of the spleen, snake bite, rheumatism, leprosy, eczema, ringworm (Basu and Walia, 1994), as well as anxiety, epilepsy, bronchitis, asthma, irritable bowel syndrome and gastric ulcers (Shakoor et al., 1994).

The plant is a perennial creeping herb and commonly grows in damp and marshy places throughout South Asia up to an altitude of 1320 m. The plant has small, white flowers with four or five petals. The plant is also a very popular aquarium plant due to its appearance and adaptability under slight to moderate brackish conditions.

Due to its medicinal properties, several regeneration protocols have been reported (Tivari and Singh, 2008; Sharma et al., 2010; Vijaykumar et al., 2010). In Turkey, the plant is mainly used as an aquarium plant and the aim of the present study was to develop a reliable protocol for this highly important medicinal, rock garden and ornamental aquarium plant and an acclimatization protocol of tissue-cultured plants under aquatic conditions.

MATERIALS AND METHODS

The water hyssop plants were obtained from local traders of aquatic plants in Karaman province of Turkey and were identified by the plant taxonomists in the Department of Biology, Kamil Ozdag Faculty of Science, Karamanoglu Mehmetbey University, Yunus Emre Campus, Karaman, Turkey. Plant twigs with 4-5 nodes with attached leaves were first washed with tap water for 5 min and then surface sterilized with 40% (v/v) H_2O_2 for 10 min. Thereafter, the plants were rinsed three times with sterilized redistilled water by continuous stirring for 5 min. The leaves were detached from the twigs and the top three internodes were isolated under aseptic conditions and cultured on MS (Murashige and Skoog, 1962) medium supplemented with 30 g sucrose per liter and solidified with 0.65% agar, devoid of plant growth regulators for 2 weeks to obtain contamination-free explants. Thereafter, internodes and leaf explants were cultured on MS medium containing different combinations of BA (0.25, 0.50, 1.0, mg/l) and NAA (0.25, 0.50, 1.0, mg/l) (Table 1). All culture media were 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 a 16 h light photoperiod (4000 lux) using white LED (Light Emitting Diodes) lights.

After 6 weeks of culture, the regenerated shoots were rooted on agar-solidified MS rooting medium containing 0.25, 0.50 and 1.0 mg/l IBA in Magenta GA7 vessels. After 3 weeks the adhering gel was removed from the root zone of *in vitro*-grown rooted plants and they were acclimatized at different pH values (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) in jars containing water. The jars were kept open for acclimatization in a growth room under white LED lights at temperature range of 26 ± 2°C. After 4 weeks of culture, data regarding plant growth were recorded (Table 2).

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 LSD or t test. Data given in percentages were subjected to arcsine transformation (Snedecor and Cocharan, 1967) before statistical analysis.

RESULTS

The study presents the efficient adventitious shoot regeneration from different internodes and leaf explants of the medicinal aquatic plant, water hyssop, cultured on MS media containing different combinations of BA and NAA followed by rooting and acclimatization.

Callus induction and shoot regeneration started within one week of culture and recorded 100%. However, the explants behaved variably to BA-NAA concentrations in terms of callus and shoot induction. Callus and shoot induction started within one week with clear shoots after 2 weeks of culture (Fig 1a,b,c). Some explants at first induced the callus (Fig 1a) then shoot on MS medium containing a higher concentration of NAA (1 mg/l), whereas, some explants first induced shoots then callus on MS medium containing lower or equal concentrations of NAA to BA (Fig 1b). On the other hand, both callus initiation and shoot induction started simultaneously irrespective of growth regulators in the culture medium on leaf explant (Fig 1c). Clear shoot regeneration with more or less calli on the explants were recorded after 3 weeks of culture and data regarding the frequency of shoot regeneration and calli, mean number of shoots

Table 1. Effects of various concentrations of BA-NAA on number of shoots per explant of *B. monnieri*

BA	NAA	1. internode	2. internode	3. internode	Leaf
0.25	0.25	21.89a	21.22a	23.11a	21.77a
0.25	0.50	15.00b	10.89d	11.89b	12.07b
0.25	1.00	11.00d	10.00e	9.00c	10.60b
0.50	0.25	13.00c	12.11b	9.00c	13.47b
0.50	0.50	8.00f	8.89f	7.89d	11.13b
0.50	1.00	8.89e	6.89g	7.89d	9.93b
1.00	0.25	13.11c	11.89b	9.56c	12.80b
1.00	0.50	11.11d	11.44c	9.56c	14.53b
1.00	1.00	9.00e	10.00e	8.33d	8.40b

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

Table 2. Effects of various concentrations of BA-NAA on mean shoot length of *B. monnieri.*

BA	NAA	1. internode	2. internode	3. internode	Leaf
0.25	0.25	3.06b	2.89ab	3.04b	3.02a
0.25	0.50	3.42a	1.69g	2.80c	2.62ab
0.25	1.00	2.30f	2.06f	2.30f	2.10b
0.50	0.25	2.40e	2,37d	3.19a	2.58ab
0.50	0.50	2.70c	2,96a	2.73cd	2.42ab
0.50	1.00	1.84g	1,29h	1.27g	2.08b
1.00	0.25	2.61d	2,87ab	2.63e	2.67ab
1.00	0.50	1.23h	2,24e	2.69de	2.42ab
1.00	1.00	2.56d	2,74c	1.22g	2.49ab

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

Table 3 Effects of Different pH on acclimatization and growth of *B. monnieri.*

pH	Plant height			Number of internodes		
	At acclimatization	After 1 month of acclimatization	% change	At acclimatization	After 1 month of acclimatization	% change
4.0	4.0	5.17c	29.25	4.67	5.67bc	21.41
5.0	4.0	7.33b	83.25	5.00	6.67b	33.34
6.0	4.0	7.33b	83.25	4.67	6.37b	42.83
7.0	4.0	7.70b	92.50	5.00	7.63a	52.60
8.0	4.0	8.67a	116.75	5.00	8.13a	62.60
9.0	4.0	5.50c	37.50	4.67	5.67bc	21.41
10.0	4.0	5.17c	29.25	4.67	5.33c	14.13

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

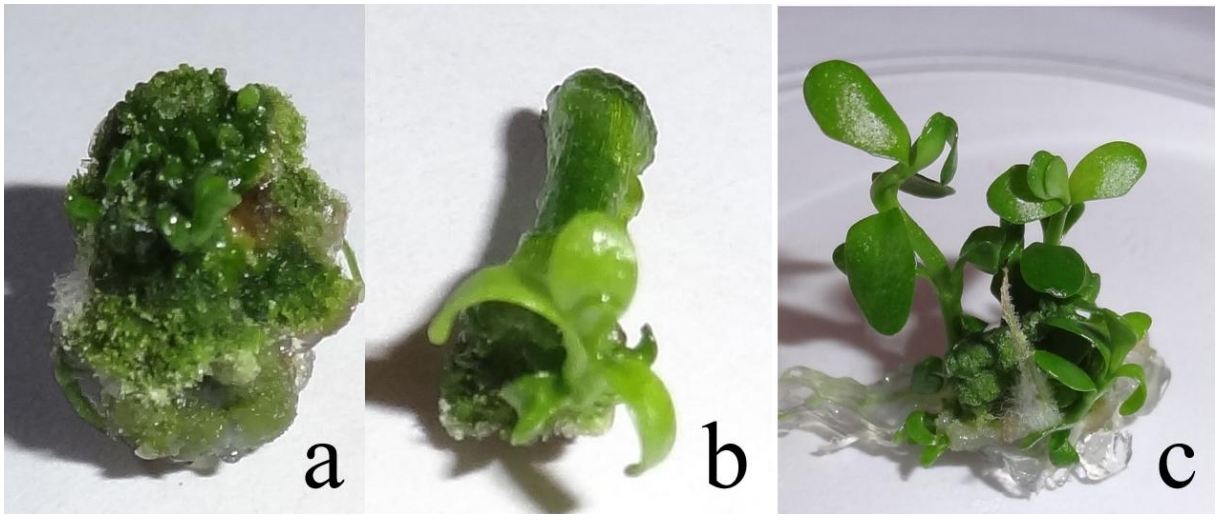

Fig. 1. Callus induction and shoot initiation after 2 weeks of culture (a) callus induction, (b) shoot initiation from internode explant (c) callus induction and shoot initiation from leaf explant.

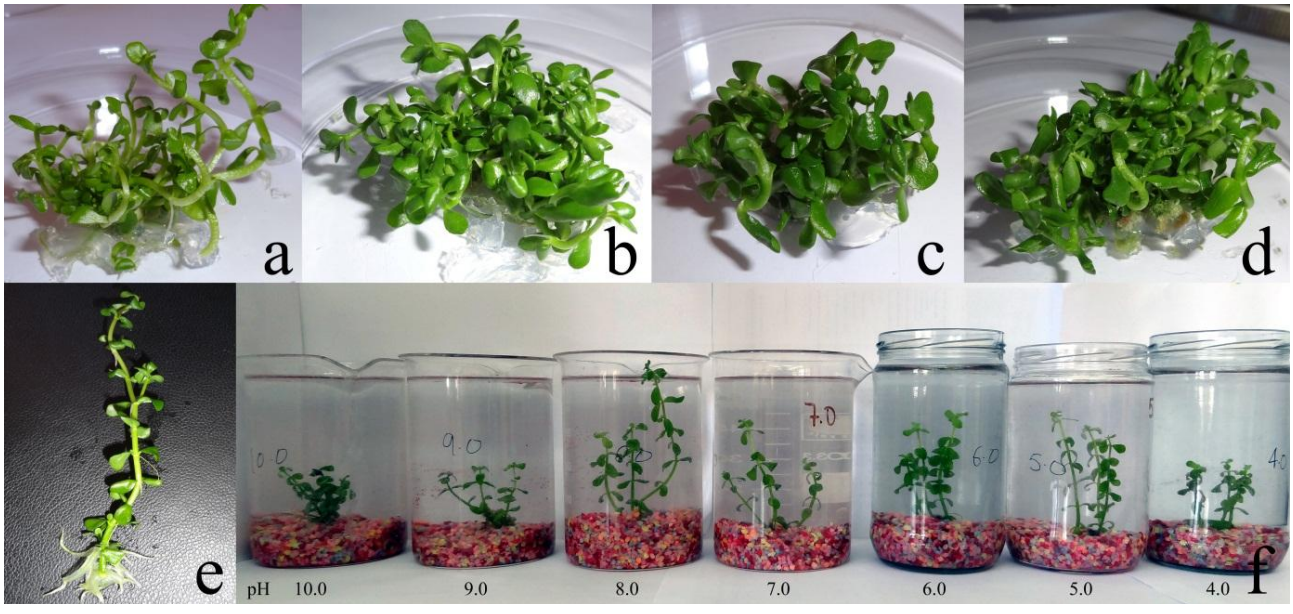

Figure 2 Adventitious shoot regeneration, rooting and acclimatization of *B. monnieri*. Shoot regeneration from the 1st (a); 2nd (b); 3rd internodes (c) and leaf explants (d) after 6 weeks of culture of an *in vitro* rooted plantlet (e) and 1 month old acclimatized plants (f) in jars containing water at different pH levels.

per explant and mean shoot length, were taken after 6 weeks of culture.

Results showed that both internodes (Fig 2 a,b,c) and leaf explants (Fig 2d) exhibited a similar trend of regeneration in response to growth regulators after 6 weeks of culture. The mean number of shoots per explant was 8.89-21.89, 6.89-21.22, 7.89-23.11 and 8.40-21.77 on 1st, 2nd, 3rd internode and leaf explants, respectively (Table 1). Comparing the explants, the leaf explant generated a greater number of shoots per explant compared to the 1st, 2nd and 3rd

internode explants at all combinations of BA-NAA. Comparing the combinations of BA-NAA, the maximum number of shoots per explant on all internodes and leaf explants was observed on MS medium supplemented with 0.25 mg/l BA+0.25 mg/l NAA and recorded 21.89, 21.22, 23.11 and 21.77 on the 1st, 2nd, 3rd internodes and leaf explants, respectively (Table 1). Similarly, 0.25 mg/l NAA in the culture medium resulted in the maximum number of shoots per explant with 0.50 and 1.0 mg/l BA on all three explants used in the experiment. Results further showed that an increase of BA concentration (0.50 and 1.0 mg/l BA) with all concentrations of NAA had an inhibitory effect on the mean number of shoots per explant as compared to 0.25 mg/l BA+0.25 mg/l NAA. However, it was the higher concentration of 1.0 mg/l NAA which inhibited shoot regeneration more with all concentrations of BA in the culture medium on all internode explant. The minimum number of 6.89 shoots per explant was recorded on MS medium supplemented with 0.50 mg/l BA+1.0 mg/l NAA (Table 1).

Results on mean shoot length showed variable response of internode explants to the various combinations of growth variants. However, the shoot length of the leaf explant showed a similar response and produced shoots of more than 2.0 cm in length (Table 2). Shoot length ranged 1.23-3.42, 1.29-2.89, 1.22-3.04 and 2.08-3.02 cm on 1st, 2nd, 3rd internodes and leaf explants respectively (Table 2). Both longer (3.42 cm) and shorter shoots were recorded from the 1st internode on MS medium supplemented with 0.25 mg/l BA+0.50 mg/l NAA and 1.00 mg/l BA+0.50 mg/l NAA, respectively (Table 2).

Well developed *in vitro*-regenerated shoots above 1.0 cm in length from all culture media were isolated and cultured on MS media supplemented with variable concentrations of IBA. Root initials started within 3-6 days and 100% rooting was recorded after 3 weeks of culture. At a low concentration of IBA in the culture medium, root initiation was faster and gradually reduced with an increase of IBA concentration. After 2 weeks of culture, callus induction started that was followed by secondary shoots initiation. After 3 weeks of culture, *in vitro* rooted plantlets (Fig 2e) were acclimatized in jars containing water with variable pH levels (Fig 2f). Plants with similar height (average of 4.0 cm) and number of internodes (4.67-5.0) were selected for acclimatization. After 4 weeks, 100% plants were adapted and data regarding plant height and number of internodes were taken followed by measuring the percent change.

Data regarding plant height revealed a 29.25-116.75% change after 1 month of culture (Table 3). Maximum plant height was recorded at pH 8.0 with 116.75% change in plant height. However, pH levels above 8.0 had inhibitory effects on plant height and ranged from 29.25-37.50. Similarly, a low pH value of 4.0 also inhibited plant growth. As with plant height, pH values had significant effects on the number of internodes and 14.13-62.60% change was recorded after one month of culture (Table 3). Maximum % change (62.60%) was recorded for the pH value of 8.0 followed by strong inhibition caused by increased pH. Results also showed that plants could be established in slightly acidic to slightly alkaline media. High acidity and alkalinity hinders the growth of *in vitro*-grown plantlets.

DISCUSSION

The present study presents an efficient and reliable protocol for adventitious shoot regeneration and acclimatization of an important medicinal and ornamental aquarium plant, water hyssop, using different internode and leaf explants in different combinations of BA-NAA.

Of all the explants, complete (100%) callus induction and shoot regeneration showed the greatest response to the growth regulators. Vijaykumar et al. (2010) reported 30.0-95.0% and 50.0-95.0% shoot regeneration frequency of *B. monnieri* cultured on BA and TDZ, respectively, under fluorescent light. However, explants behaved variably to the presented conditions of growth in the culture medium. Although callus and shoot induction was recorded on all explants on all culture media, it was the combination of BA-NAA that directed the regeneration process. A

higher concentration of NAA in the culture medium led to callus induction followed by shoot induction. On the other hand, equal or lower concentrations of BA to NAA resulted in shoot induction followed by callus induction. Sancak et al., (2000) reported the reduction in shoot regeneration frequency at an equal or a high NAA to BA concentration in the media on immature cotyledon leaf explants. Similarly, Celiktas et al. (2006) also reported significant effects of NAA concentration on callus-induced regeneration on the internodes, young leaves, apical-axillary meristems, petioles and immature inflorescences in sainfoin.

Results on the mean number of shoots per explant showed that all explants responded in similar way to all growth variants. These results are contradictory to the findings of Özcan et al. (1996), who reported the variable response of different explants to adventitious shoot regeneration in sainfoin. However, leaf explants was more responsive than all internode explants. Among the internodes, the 1st internode generated more shoots per explant on all combinations of BA-NAA compared to the 2nd and 3rd nodes; this might be due to the age and relatively higher number of young and actively dividing cells, and is in agreement with Celiktas et al. (2006).

The results also emphasize that the cytokinin-auxin ratio is important for maximum shoot regeneration. However, it was the presence of NAA in the culture medium that ultimately controlled the shoot regeneration behavior. A lower concentration of NAA (0.25 mg/l) with all concentrations of BA in the culture medium resulted in more shoots per explant due to early shoot induction and delayed callusing as compared to higher concentrations of NAA (0.50 and 1.0 mg/l). Özgen et al. (1998) reported higher shoot regeneration from a high cytokinin to auxin ratio in sainfoin. A higher concentration of NAA promoted callus induction at an early stage on all explants that delayed the shoot induction and in turn decreased shoots per explant. Results also emphasize the importance of BA concentration on the number of shoots per explant that decreased with an increase of BA concentration in combination with NAA. Sharma et al. (2010) reported a relatively low amount of BA for the maximum number of shoots per explant in *B. monnieri*.

Results on mean shoot length showed that both growth regulators and explants exerted variable effects. Vijaykumar et al. (2010) reported an increase in shoot length with an increase in BA and TDZ concentration in the culture medium using the leaf explant of *B. monnieri*. However, it was the leaf explant that responded differently to internodes. The variable response of calli and shoot regeneration behaviors affected by the combination of BA+NAA at early stage also significantly affected the mean shoot length and resulted in variable shoot length. It was also observed that explants with greater frequency of callusing resulted in relatively shorter shoots as compared to explants with greater shoot regeneration frequency.

The results on rooting showed the greater response of IBA at all concentrations and recorded 100%. Tivari and Singh (2008), Ceasar et al. (2010) and Sharma et al. (2010) also used IBA for successful rooting in *B. monnieri*. However, plants need less IBA at the early stage and higher concentrations of IBA in the culture media promoted callus induction and secondary shoot initiation. Multiple shoot induction by IBA is an unexplained phenomenon and has been previously reported in many legumes such as cowpea (Aasim et al., 2010), chickpea (Aasim et al., 2011) and lentil (Aasim et al., 2011).

The acclimatization experiments by other researchers showed that the plants can be established easily in soil substrate (Tivari and Singh, 2008; Sharma et al. 2010). However, there is no report on successful acclimatization of *in vitro*-regenerated plants in water directly. However, Öztürk et al. (2004) successfully acclimatized *Ludwigia repens* in an aquarium. Results showed 100% establishment of plants in jars containing water at variable pH. However, the plant growth was clearly affected by pH. Results showed that the plant needs slightly acidic to alkaline medium for proper growth. However, a normal to slightly alkaline medium is the most suitable for obtaining maximum height and increasing the number

of internodes. It was also found that highly acidic or alkaline media are not good for plant growth.

The establishment of a successful regeneration, rooting and acclimatization protocol in water of *B. monnieri* is an important step for the application of biotechnological tools to multiply the plant for multiple uses as an ornamental plant. The direct acclimatization of plants in water also opens the window for the commercial propagation of the plant for aquaria and for use in water systems to prevent water pollution. The protocol can also facilitate as a base for the extraction of medicinally important compounds from this important aquatic plant.

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