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INFLUENCE OF LIGHT-EMITTING DIODES AND BENZYLAMINOPURIN ON ADVENTITIOUS SHOOT REGENERATION OF WATER HYSSOP (*BACOPA MONNIERI* (L.) PENNELL) *IN VITRO*

Mehmet Karataş[1], Muhammad Aasim[1,*] and Muraz Dazkirli[2]

[1] *Department of Biotechnology, Faculty of Science, Konya Necmettin Erbakan University*, Konya, Turkey

[2] *Department of Biology, Kamil Ozdag Faculty of Science, Karamanoglu Mehmetbey University, Yunus Emre Campus*, 70200, Karaman, Turkey

***Corresponding author:** mshazim@gmail.com

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Abstract: Water hyssop (*Bacopa monnieri* (L.) Pennell) is a medicinal plants. Its upper and lower halves of leaf explants were incubated in Murashige and Skoog (MS) medium supplemented with 0.25, 0.50 and 1.0 mg/L benzylaminopurine (BA) for 8 weeks; the explants were exposed to white (W) and red and blue (R and B, respectively) light-emitting diodes (LEDs), at 4:1, 3:1, 2:1 and 1:1 R and B light ratios, respectively. Shoot regeneration (100%) was achieved from all explants at all applied concentrations of BA and LED types. All explants showed different BA concentration requirements for regeneration of the maximum number of shoots. Longer shoots were obtained on medium with 0.25 mg/L BA. The W LED lighting system was found to be more effective for regenerating the maximum number of shoots (26.11) per explant (on the upper half of the leaf). Conversely, longer and shorter shoots were generated under 1:1 R:B and W LEDs, respectively. The number of shoots per explant ranged from 9.67-24.0 (full leaf), 6.33-25.92 (lower half of the leaf) and 7.33-27.33 (upper half of the leaf), respectively, in response to BA and LED light. Shoot length ranged from 0.94-1.90 cm (full lamina), 0.70-2.11 cm (lower half of the leaf) and 0.93-1.83 cm (upper half of the lamina) in response to BA and LED lifght. Regenerated shoots were successfully rooted using indole-3-butyric acid (IBA) and acclimatized in the aquarium provided with tap water.

Key words: adventitious; light-emitting diodes (LEDs); leaf explants; shoot regeneration; water hyssop

INTRODUCTION

Water hyssop (*Bacopa monnieri* (L.) Pennell) or Brahmi is a well-known Indian plant grown in damp and marshy areas [1,2]. It is one of the most important medicinal plants [3], which contains medicinally important alkaloids, saponins, flavonoids and bacosides [4], used as cardiac or brain tonic, for treating anxiety and epileptic disorders [5,6], for their diuretic, analgesic, anti-inflammatory and antipyretic activities [7,8], treatment of snakebites, spleen enlargement, rheumatism, ringworm, leprosy, eczema [9] asthma, irritable bowel syndrome, gastric ulcers and bronchitis [10]. Because of overexploitation the Water hyssop is a threatened and endangered species [1,11]. High demand for this plant is a challenge for researchers to develop new regeneration protocols in order to conserve plants and offer an alternative routes of production, e.g. by *in vitro* propagation[3,12-16].

Plant tissue culture provides an alternate way to propagate plants under controlled conditions and also makes it possible to alter metabolite concentrations using modern biotechnological techniques such as genetic transformation, modifying growth media or culture conditions. Lighting systems or photoperiod are one of the major components of successful regeneration protocols *in vitro*, also providing an opportunity to alter secondary metabolite concentrations in plants [17,18]. Light-emitting diodes (LEDs) offer an alternative lighting system available in different colors. The advancement in LED technology, low prices, long

life, low electricity costs and availability make it possible to use this technology for *in vitro* propagation studies. LEDs can alter plant metabolite concentrations both *in vitro* [19,20] and *in vivo* [21-23] by using monochromatic LEDs or in different combinations.

The use of LEDs for *in vitro* propagation research is very limited and previous work has mainly described studies on ornamental, bulbous plants [24,25] and fruits, such as strawberry [26]. There is no report about the effect of different red and blue LED combinations on aquatic plants. The aim of the present study was to show the potential of different combinations of red (R) and blue (B) LEDs, as compared to white (W) LEDs, and their effect on shoot regeneration obtained from different leaf explants of water hyssop.

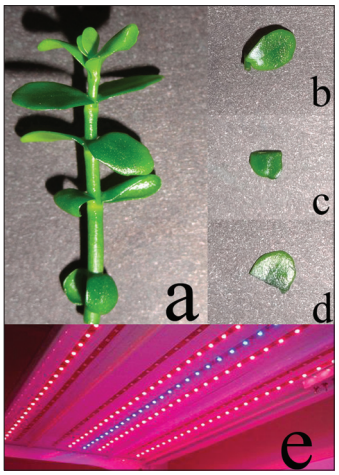

Fig. 1. Leaf explants. Leaf explants isolated from the stem (**a**); sterile stem (**b**); full leaf explant (**c**); lower half leaf explant (**d**); upper half leaf explant (**e**) exposed to the R:B LED lighting system.

MATERIALS AND METHODS

Plants

Plants were obtained from the Hydrobiology Laboratory of Karamanoglu Mehmetbey University, Department of Biology. Stems, 4-6-cm long, with 4-5 nodes and leaves were sterilized using the protocol of Karataş and Aasim [27]. After sterilization, individual stems were cultured on Petri dishes containing 0.65% agar solidified MS [28] medium enriched with 30 g/L sucrose without plant growth regulators for two weeks. Thereafter, leaf explants were taken by cutting them from the stems (Fig. 1a) under aseptic conditions. The full leaf (Fig. 1b) was cut horizontally into an upper (Fig. 1c) and lower half (Fig. 1d) leaf explants for adventitious shoot regeneration. All explants were cultured in Magenta GA7 vessels containing MS medium with 0.25, 0.50 and 1.0 mg/L benzylaminopurine (BA) and 30 g/L sucrose and 6.5 g/L agar. Thereafter, they were incubated under R:B LED combinations (4:1, 3:1, 2:1 and 1:1, respectively), along with W LEDs (Fig. 1e).

One cm long *in vitro* regenerated shoots were separated carefully from the explant, transferred to medium with 0.25, 0.50, 0.75 and 1.0 mg/L IBA in Magenta GA7 vessels and cultured under white LEDs for 4 weeks, for rooting. Rooted plantlets was removed from the medium, cleaned carefully under running tap water without damaging the roots, and immediately transferred to the aquaria containing tap water and commercial aquarium sand. Experiments were performed twice with six replicates each. The pH value of all nutrition media used in the study was adjusted to 5.8 prior to autoclaving at 118 kPa atmospheric pressure at 120°C for 21 min. Explants were incubated for a 16-h light photoperiod using R:B and W LEDs.

Reagents

All chemicals used in the study were purchased from Duchefa Biochemie, Germany.

Data analysis

Data pertaining to shoot regeneration frequency (%), shoots per explant and shoot length were scored after 8 weeks of culture and subjected to one-way ANOVA statistical analysis using SPSS20 for Windows. Post hoc tests were performed using Duncan's multiple range test (DMRT), and arcsine transformation was used for data provided in percentages [29].

RESULTS

Direct shoot induction started within 8-10 days from the margins of leaf explants (Fig. 2a and b) followed by shoot bud induction and shoot regeneration within three weeks (Fig. 2 c). Shoot buds were grown to normal size. After eight weeks of growth *in vitro*, multiple adventitious shoot regeneration was observed on all leaf explants (Fig. 2 d), Thereafter, data regarding shoot regeneration frequency, shoots per explant and mean shoot length were recorded. Shoot regeneration was recorded on all explants (100%), irrespective of LED type or combination and BA concentrations. Data regarding shoots per explant and mean shoot length were analyzed. Results showed statistically significant effects of BA ($p \leq 0.01$), LEDs ($p \leq 0.01$), and the interactive effects of BA and LEDs (BA × LEDs) of individual explants ($p \leq 0.01$).

BA concentrations in the culture medium showed clear bearings on shoots per explant and shoot length in all explants. The number of shoots per explant obtained from the full (Table 1) and upper half leaf (Table 3) showed no statistically significant difference with regard to BA concentration, unlike the number of shoots obtained from the lower half leaf (Table 2), which displayed statistical differences according to BA concentration. The number of shoots per explant was recorded as 16.13-16.55 (Table 1), 16.5-18.62 (Table 2) and 17.78-19.25 (Table 3) for the full, upper half and lower half of the leaf explants, respectively. The upper and lower leaf explants had better potential for shooting than the full leaf when the explants were cultured

Table 1. Adventitious shoot regeneration from full leaf explant of water hyssop in response to exposure to different concentrations of BA and LED lighting.

BA (mg/L)	LED Light (R:B)	Number of shoots per explant	Shoot length (cm)
0.25	-	16.13^{ns}	1.69a
0.50	-	16.32	1.51ab
1.0	-	16.55	1.34b
-	**4:1**	15.64b	1.51b
-	**3:1**	14.06b	1.42b
-	**2:1**	16.83b	1.50b
-	**1:1**	13.58b	1.88a
-	**W**	21.56a	1.26b
0.25	**4:1**	12.67cd	1.92a
	3:1	15.67bcd	1.87abc
	2:1	14.50bcd	1.50abcd
	1:1	13.83bcd	1.90ab
	W	24.00a	1.27de
0.50	**4:1**	17.08abcd	1.43bcd
	3:1	16.83abcd	1.43bcd
	2:1	16.00bcd	1.47abcd
	1:1	10.33d	1.90ab
	W	21.33ab	1.33de
1.0	**4:1**	17.17abcd	1.19de
	3:1	9.67d	0.94e
	2:1	20.00abc	1.53abcd
	1:1	16.58abcd	1.83abc
	W	19.33abc	1.18de

Means followed by different small letters within columns are significantly different ($p<0.01$)

Table 2. Adventitious shoot regeneration from lower half leaf explant of water hyssop in response to exposure to different concentrations of BA and LED lighting.

BA (mg/L)	LED Light (R:B)	Number of shoots per explant	Shoot length (cm)
0.25	-	16.15b	1.68a
0.50	-	18.62a	1.46b
1.0	-	18.32a	1.36b
-	**4:1**	17.42a	1.38c
-	**3:1**	20.20a	1.23c
-	**2:1**	17.56a	1.69b
-	**1:1**	13.78b	1.99a
-	**W**	19.53a	1.21c
0.25	**4:1**	12.92de	1.37e
	3:1	22.67ab	1.38e
	2:1	16.50bcde	2.05ab
	1:1	6.33f	2.00abc
	W	22.33ab	1.58cde
0.50	**4:1**	25.92a	1.43de
	3:1	23.00ab	1.45de
	2:1	18.33bcd	1.61bcde
	1:1	15.17cde	2.11a
	W	10.67ef	0.70f
1.0	**4:1**	13.42cde	1.34e
	3:1	14.93cde	0.86f
	2:1	17.83bcd	1.40de
	1:1	19.83abc	1.87abcd
	W	25.58a	1.33e

Means followed by different small letters within columns are significantly different ($p<0.01$)

Table 3. Adventitious shoot regeneration from upper half leaf explant of water hyssop in response to exposure to different concentrations of BA and LED lighting.

BA (mg/L)	LED Light (R:B)	Number of shoots per explant	Shoot length (cm)
0.25	-	19.25ns	2.02a
0.50	-	18.00	1.58b
1.0	-	17.78	1.37b
-	**4:1**	19.94b	1.76b
-	**3:1**	17.44bc	1.37c
-	**2:1**	15.75c	1.54bc
-	**1:1**	12.47d	2.13a
-	**W**	26.11a	1.47bc
0.25	**4:1**	15.83de	1.68bcde
	3:1	19.25cd	1.66bcde
	2:1	12.25ef	2.03b
	1:1	21.58bc	2.83a
	W	27.33a	1.92bc
0.50	**4:1**	17.83cd	1.78bcde
	3:1	18.00cd	1.28cdef
	2:1	19.33cd	1.67bcde
	1:1	8.50f	1.73bcde
	W	26.33ab	1.43bcde
1.0	**4:1**	26.17ab	1.82bcd
	3:1	15.08de	1.18def
	2:1	15.67de	0.93f
	1:1	7.33f	1.83bcd
	W	24.67ab	1.07ef

Means followed by different small letters within columns are significantly different ($p<0.01$)

with the addition of BA. BA caused similar effects on shoot length. Shoot length ranged from 1.34-1.69 cm (Table 1), 1.36-1.68 cm (Table 2) and 1.37-2.02 cm (Table 3) for full, lower and upper half leaf explants, respectively. In general, all explants regenerated longer shoots on MS medium with 0.25 mg/L of BA.

LED type and the combinations of R and B lights exhibited statistically significant differences on the number of shoots per explant and shoot length. The number of shoots per explant ranged from13.58-21.56 (Table 1), 13.78-20.20 (Table 2) and 12.47-26.11 (Table 3) for full, lower half and upper half leaf explants, respectively. The W LED lighting system was more efficient for regenerating the maximum number of shoots per explant compared to the R:B LEDs, irrespective of explant type. The maximum number of shoots per explant (26.11) was scored on the upper half leaf exposed to W LEDs. The ratio of R:B LEDs played a significant role in shoot regeneration behavior and the 1:1 R:B lighting system was least responsive, with the lowest number of shoots per explants, irrespective of explant type. Results on the shoot length of all explants exposed to different LEDs showed that longer shoots from all explants were regenerated under 1:1 R:B LEDs. The shoot length of full, lower half and upper half leaf explants was recorded as 1.26-1.88 cm (Table 1), 1.21-1.99 cm (Table 2) and 1.47-2.13 cm (Table 3), respectively.

A combination of BA and LEDs had different effects on the number of shoots per explant and shoot length compared to their separate effects. The number of shoots per explant ranged from 9.67-24.0 (Table 1), 6.33-25.92 (Table 2) and 7.33-27.33 (Table 3) for full, lower half and upper half leaf explants, respectively. In general, full and upper half leaf explants responded similarly and reached the maximum number of shoots per explant under the W LED lighting system with all BA concentrations used in the study. On the other hand, lower half leaf explants yielded the maximum number of shoots (25.92) with 0.50 mg/L BA and 4:1 R:B LEDs, and was statistically similar to the other combinations of BA and 4:1 R:B LEDs. The minimum number of shoots per explant (6.33) was observed on lower leaf (Table 2) explants grown on 0.50 mg/L BA and 1:1 R:B LEDs. Shoot length in response to BA and LEDs ranged from 0.94-1.90 cm, 0.70-2.11 cm and 0.93 to 1.83 cm from for shoots regenerated from full, lower and upper half leaves (Table 1), respectively.

All explants regenerated longer shoots in response to different combinations of BA and LEDs, with the longest shoots from full (1.92 cm), lower half (2.11 cm) and upper half (2.83 cm) leaves scored from 0.25 mg/L BA and 4:1 R:B LEDs, 0.50 mg/L BA and 1:1 R:B LEDs and 0.50 mg/L BA and 1:1 R:B LEDs, respectively. However, they were not statistically different from other combinations. Shorter shoots regenerated from all explants showed different combinations of BA and LEDs interactions, which also showed statistical insignificant effects with each other. In general, the shortest shoots (0.94 cm) from full leaf explant were

recorded under 1.0 mg/L BA and 3:1 R:B LEDs (Table 1). Shorter shoots (0.70 cm) were regenerated on lower half leaf under 0.50 mg/L BA and W LEDs. (Table 2), and on the upper half leaf shorter shoots (0.93 cm) were regenerated under 0 mg/L BA and 2:1 R:B LEDs. Our results further revealed that statistically different shoot lengths could be noticed when a shoot was regenerated from lower and upper leaf explants (Tables 2 and 3). When LEDs R:B (1:1 and 2:1) was used for shoot regeneration, shoot length was greater after exposure to the combination with 0.25 and 0.5 BA in nutrition medium for shoots regenerated from lower half explants. Shoots regenerated from upper half leaf explants had greater length when LED R:B (1:1) was used, but only in combination with 0.25 BA in the medium.

MS medium enriched with 0.25-1.0 mg/L IBA was used for rooting and acclimatization of *in vitro* regenerated shoots during four weeks under white LEDs. Roots start to show after two weeks and rooting frequency reached a maximum (100%) after four weeks of culture. Rooted plantlets (Fig. 2e) were carefully removed from the rooting medium and cleaned under tap water. Thereafter, plantlets were directly placed in the aquaria provided with sand and tap water with a pH of ~8.0. In the aquaria, plants survived and acclimatized easily in the presence of other plants and fish, gaining in mass and producing new leaves (Fig. 2 f) within a few months.

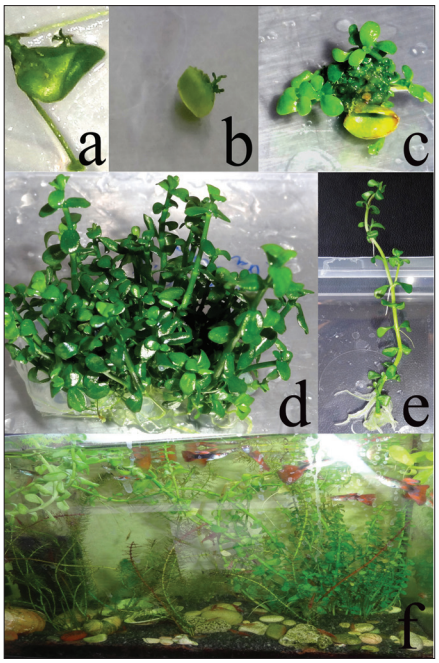

Fig. 2. Adventitious shoot regeneration from leaf explants. **a,b** – shoot initiation from margins of the leaf after 8-10 days; **c** – multiple shoot induction; **d** – leaf explant after 3 and 8 weeks of culture; **e** – rooted plantlet; **f** – acclimatized plant in the aquarium after two months.

DISCUSSION

The present study provides results about adventitious shoot regeneration on different leaf explants (full, upper and lower half leaf) of water hyssop in response to BA and different LED lighting systems. The study presents the first report about LED lighting systems for *in vitro* regeneration of aquatic plant. Explants such as shoot tips, internodes, meristematic nodes and leaves, are commonly used for *in vitro* regeneration of water hyssop. Among these explants, the leaf explant is the most potent explant [3,13,16,27]. Direct shoot initiation from leaf margins within a short time on leaf explants of the protoplast culture of water hyssop [2], confirmed previous findings [3, 27] that 8-10 days were required for shoot induction. Multiple shoot buds appeared after three weeks on all explants in response to BA and LEDs with a regeneration rate of 100% after eight weeks. Maximum shoot regeneration (100%) of water hyssop using full leaf explants has been reported [1,27]. In contrast, lower percentages of shoot regeneration (41.1-80.5% and 80-100%) were obtained from the leaf explant of water hyssop using different BA concentrations [13,30]. However, previous results, as well as results from our laboratory, have revealed the importance of BA, LEDs and BA together with exposure to LED combinations on shoot regeneration, their number and length.

Our results revealed the importance of explant type and BA concentration for obtaining the maximum number of shoots per explant in water hyssop, confirming previous findings. [3] also obtained on

different leaf explants. Results further confirmed the importance of cutting the leaf into an upper and lower half, which reached twice the number of shoots compared to the full leaf, irrespective of BA concentration. Results also confirmed the significance of low BA concentrations in the culture medium for regenerating the maximum numbers of shoot per explant. The low requirement for BA for shoot regeneration, by both lower and upper half leaf explants, might be due to the active uptake of BA from the cut surfaces of explants, which resulted in more cell division and ultimately led to early shoot regeneration. However, previous work on water hyssop indicated the need for a higher BA concentration (2.0 mg/L) [13,15,30]. On the other hand, shoot length showed a similar trend, which resulted in longer shoots when exposed to low BA concentrations. This result is in agreement with the earlier results for water hyssop, where longer shoots were obtained using up to 0.5 mg/L of BA [27,31]. However, several authors [13,15] have emphasized the need for a higher BA concentration (2.0 mg/L) in the culture to for obtain longer shoots of water hyssop regenerated from leaf explants. Results further highlighted the negative effects of increased BA concentration (>1.0 mg/L) on shoot length, confirmed by previous findings [6,27].

LEDs provides an alternate way of lighting with specific light intensities and have been reported in *in vitro* callus induction, protocorm-like body formation [32,33] and *in vitro* propagation of important plants [34,35]. LED types and their combinations significantly affected the shoot regeneration of all explants. Results revealed that W LEDs were more potent lighting for multiple shoot regeneration of water hyssop, irrespective of explant type. However, earlier works on different plant species showed that a R:B LED ratio had a better effect on shoot regeneration. Improved shoot proliferation of cymbidium orchid under 75%:25% (R:B, respectively) LEDs has been reported [25], as well as enhanced *in vitro* micropropagation and growth of *Calanthe* hybrid plantlets under B and R LED lights [34]. The difference in the results might be due to different explant type, species and use of fluorescent lamps or monochromatic LEDs in these studies. Results related to shoot length also indicated the connection between shoot length and type of LED. W LEDs were least effective, while 1:1 R:B LEDs were the best combination for enhancing shoot length of a certain explant type, and 1:1 R:B LEDs were the most suitable for the growth of upland cotton plantlets *in vitro* [36]. In contrast longer shoots of *Zantedeschia albomaculata* regenerated under R or B light LEDs [37], while longer shoots of *Chrysanthemum* were grown under B LEDs [38].

Light and cytokinins control the growth, development and physiological processes of plants. To date, very little is known about the effect of LEDs in combination with BA on *in vitro* shoot regeneration. Comparing the interactive effects of BA and LEDs, the response of all explants was different and required different combinations of BA and LEDs for obtaining the maximum and minimum number of shoots per explant and shoot length of individual explants. In general, W LEDs were found to be more suitable for regenerating the maximum number of shoots with all concentrations of BA. We assume that W LEDs provided light at wavelengths ranging from violet to red light, with provision of UV and IR light, which helped to induce an increase in cell division, with more shoot bud induction and shoot proliferation in the presence of BA in the culture medium. Another possibility is increased endogenous cytokinin production in response to light [39]. However, these results do not support earlier research [26] in which the highest number of shoots was obtained under B, R or orange LEDs as compared to fluorescent and Growlux in strawberry, irrespective of the concentration of BA. In contrast, the shoot length of each explant responded variably to the interaction of BA and LED. The variable response to BA and LEDs might be due to the interaction between cytokinin and light, but their interaction is still unknown [40]. Results revealed that R:B LEDs were superior for gaining longer shoots compared to white LEDs. This might be due to the need for a specific wavelength [36,37], and LEDs triggered the photomorphogenic pigments, which are responsible for photoreception and regeneration [24]. Enhanced plant growth and development under R:B LEDs due to an increased net photosynthetic rate was reported [41]. It is supposed that shoot length can be enhanced

or inhibited by using different R:B LED combinations, which shows the synergistic interactions between B and R light receptors and phytochrome depending on the type of explant [38]. The B light photoreceptor class of cryptochromes controls different plant growth factors in conjunction with R/far-R phytochrome photoreceptor classes [42].

Rooting and acclimatization of *in vitro* regenerated shoots of aquatic plants is of great importance for the development of a successful *in vitro* regeneration protocol. In the present study, plants were rooted and established in aquaria, as reported earlier [27,31]. Our study emphasizes the successful use of different LEDs for *in vitro* regeneration of aquatic water hyssop. In addition, the efficacy of different leaf explants used in the study was also tested and the obtained results showed that two-fold more plants regenerated from leaves cut into two pieces in comparison to the whole leaf. Our results also suggested that further cutting of leaf into small pieces might increase shoot regeneration. These findings can be used for further studies into the effect of LED lighting systems on secondary metabolite concentrations during callus induction or whole plant regeneration.

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