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Micropropagation of *Baliospermum montanum* (Willd.) Muell. Arg.—A threatened medicinal plant

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An efficient *in vitro* regeneration protocol was developed for *Baliospermum montanum* (Willd.) Muell. Arg., a wild threatened medicinal plant. The plants were regenerated from young nodal buds and shoot tips. The morphogenic frequency of shoot bud induction and shoot multiplication was significantly higher in nodal segments when compared to shoot tips. Maximum number of shoots (22.2 ± 0.84) with high frequency of shooting response (82%) was obtained in nodal explants cultured on MS medium fortified with 2.0 mg L^{-1} BAP. Maximum shoot height of 15.8 cm was achieved. Caulogenic effect of BAP was found to be significant compared to Kn. The excised shoots were cultured on MS medium with various concentrations and combinations of auxins for rooting. Maximum number of healthy rootlets (14.8 ± 2.07 cm) with 90% rooting response was observed due to a synergistic action of IBA (1.0 mg L^{-1}) and IAA (0.5 mg L^{-1}) on half strength MS basal medium. The regenerated plantlets were transferred to the natural habitat with 86.2% success.

Keywords: *Baliospermum montanum*, micropropagation, threatened plant

Introduction

Baliospermum montanum (Willd.) Muell. Arg. (Euphorbiaceae) is an important woody medicinal plant, found in Southern Western Ghats of India. Seeds and roots of this plant are used to treat jaundice, skin diseases, rheumatism, snakebite and piles. Leaves are also used to cure asthma, bronchitis and abdominal tumours[1]. Due to indiscriminate collection and overexploitation for medicinal use, this plant species has been disappearing very fast and is now put under red list category[2]. *B. montanum* can be propagated conventionally by seeds and vegetative cutting. However, non-availability of seeds due to high sterility and requirement of large amount of planting materials are major bottlenecks for conventional plant propagation. Hence, *in vitro* tissue culture method might be of great value as an alternate method to achieve rapid multiplication of this ill-fated plant species.

For *ex situ* conservation of endangered medicinal plants, propagation through shoot tip and nodal bud culture is an economic way to obtain large number of consistently uniform and true-to-type plants within a short period of time[3-6]. Although, earlier attempts have been made for *in vitro* propagation of *Baliospermum* species[7, 8], the frequency of shoot induction and shoot multiplication was very low. Hence, considerable efforts are still required to find out efficient methods of regeneration of this threatened medicinal plant. We report here a reliable efficient protocol for rapid propagation of *Baliospermum montanum* using shoot tip and nodal bud explants and successful restoration of the micropropagated plants into the natural habitat.

Materials and Methods

Plant Material and Explant Source

The plants collected from Southern-Western Ghats, India were maintained in the green house and used as the explant source. The young nodal buds and shoot tip explants were collected from a healthy mother plant for this study. The explants were excised into 1.0-1.5 cm long pieces and were washed thoroughly in running tap water for 1 h, followed by washes with Tween 20 for 5 min. For surface sterilization the explants were immersed in 70% ethanol (v/v) for 30 sec and washed well with sterile double distilled water 3 times. Eventually, the explants were exposed to 0.1% (w/v) aqueous mercuric chloride solution for 5 min and then thoroughly washed with sterile double distilled water 5 times in order to remove the mercuric chloride residues. The explants were excised into pieces (0.5-1.0 cm) and were aseptically implanted into the nutrient agar medium.

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Culture Media and Conditions

The disinfected nodal buds and shoot tips were cultured on MS medium[9] containing 3% (w/v) sucrose and 0.7% agar-agar type-1. Plant growth regulators at different concentrations were incorporated into the basal media. The *p*H of the medium was adjusted to 5.8 by 1 *N* NaOH or 1 *N* HCl before autoclaving at 1.06 kg cm^{-2} (121°C) pressure for 15 min. The cultures were incubated at 25±2°C in a culture room with 70 µmol m^{-2} s^{-1} irradiance provided by cool fluorescent tubes and were exposed to a photoperiod of 16 h and 55±5% of relative humidity(RH).

Multiple Shoot Induction and Multiplication

For shoot induction and shoot multiplication, the young nodal buds as well as shoot tips were cultured on MS medium fortified with different concentrations of BAP (0.1-3.0 mg L^{-1}) and Kn (0.5-1.5 mg L^{-1}). Both the explants were cultured on medium with either of the cytokinins. After 4 wk of incubation, well developed multiple shoots were excised into single shoots and were subcultured on fresh MS medium consisting of optimal concentration of cytokinin for further shoot elongation. 20 explants were used per treatment and all experiments were repeated twice.

Rooting and Plant Regeneration

The isolated shoots (10-15 cm) rooted when cultured on half strength as well as full strength MS medium augmented with various concentrations and combinations of auxins, like IAA (0.1-1.0 mg L^{-1}) and IBA (0.5-1.0 mg L^{-1}). Observations were taken at the end of the 4^{th} wk. Well rooted shoots were washed in sterile water and transferred to plastic cups (10 cm × 8 cm) containing sterilized mixture of sand and garden soil (1:1 v/v) covered with plastic cover. The cups were incubated at 25 ± 2ºC under cool white fluorescent light (70 µmol m^{-2} s^{-1}) with 16 h photoperiod. The plastic cover was opened after 17 d and temperature was gradually increased up to 28 ± 2ºC. The fully acclimatized plants were transferred to the greenhouse.

Experimental Design and Statistical Analysis

Experiments were set up in completely randomized design. Data were collected at the end of the 4^{th} wk from culture initiation. Data were used to obtain mean and standard deviation and ANOVA test was also performed.

Results and Discussion

The nodal bud and shoot tip explants when cultured on MS basal medium with various plant growth hormones, more than one shoot was obtained in all treatments (Fig. 1A). No caulogenic response was observed in control grown on MS basal medium. This implies the necessity for supplementation of plant growth hormones exogenously for efficient development of multiple shoots. Nodal buds showed better shoot induction and shoot multiplication efficiency than shoot tip explants. Significant differences were observed in number of shoots per explant among different concentrations of plant growth regulators.

All the concentrations of BAP and Kn facilitated shoot bud differentiation. With nodal bud explant, BAP was found to be more efficient than Kn in respect of shoot initiation (Table 1). Maximum number of shoots (22.2) was obtained from nodal bud explant on MS medium with 2 mg L^{-1} BAP (Fig. 1B),

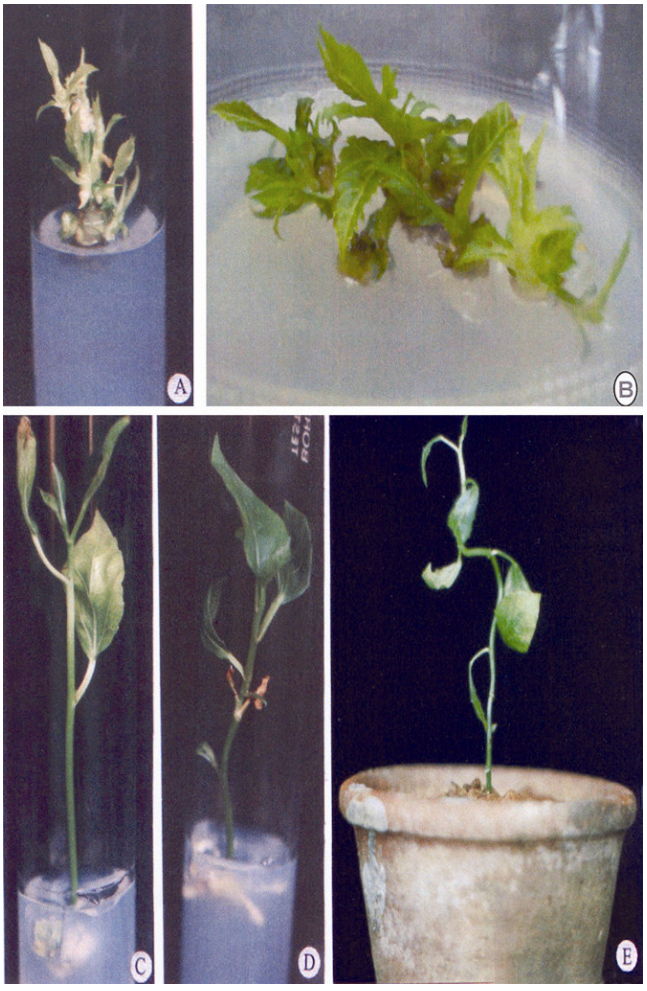

Fig. 1 (A-E)—Morphogenic response of *B. montanum* nodal bud explants: A. Shoot initiation after 17 d inoculation, B. Multiple shoot formation, C. Elongation of shoot, D. Root induction from isolated shoot, & E. *In vitro* raised plantlets transferred to pot.

Table 1—Caulogenic response of *B. montanum*

Plant growth regulators (mg L^{-1})	Nodal bud explants			Shoot tip explants		
	Shooting response (%)	No. of shoots per nodal bud explant*	Height of shootlets (cm)*	Shooting response (%)	No. of shoots per shoot tip explant*	Height of shootlets (cm)*
BAP						
0.0	0.0	0.0 ± 0.0	0.0 ± 0.0	0	0.0 ± 0.0	0.0 ± 0.0
0.1	48	2.8 ± 0.83^{b}	0.8 ± 0.14^{a}	48	3.2 ± 0.15^{c}	2.0 ± 0.62^{a}
0.5	60	6.3 ± 0.89^{cd}	3.3 ± 0.19^{d}	60	4.4 ± 0.20^{d}	3.5 ± 0.52^{b}
1.0	71	8.8 ± 1.48^{d}	1.5 ± 0.18^{b}	71	6.4 ± 0.36^{e}	2.3 ± 0.58^{a}
1.5	75	17.0 ± 1.87^{f}	7.1 ± 0.26^{f}	75	9.5 ± 0.34^{ef}	4.5 ±0.32b^{c}
2.0	82	22.2 ± 0.84^{f}	15.8 ±0.49^{g}	82	10.3 ± 0.89^{f}	7.5 ± 0.52^{d}
2.5	53	5.0 ± 0.23^{c}	4.5 ± 0.19^{e}	63	3.2 ± 0.63^{c}	4.2 ± 0.44^{b}
3.0	45	2.6 ± 0.52^{b}	2.8 ± 0.13^{c}	54	2.0 ± 0.59^{b}	4.0 ± 0.2.3^{b}
Kn						
0.0	0	0.0 ± 0.0	0.0 ± 0.0	0	0.0 ± 0.0	0.0 ± 0.0
0.1	65	1.4 ± 0.54^{a}	1.7 ± 0.11^{b}	49	1.3 ± 0.61^{a}	2.5 ± 0.35^{a}
0.5	53	2.8 ± 0.84^{b}	2.0 ± 0.23^{b}	52	2.0 ± 0.47^{b}	2.0 ± 0.38^{a}
1.0	78	3.4 ± 1.14^{b}	1.6 ± 0.30^{b}	70	3.3 ± 0.43^{c}	2.5 ± 0.21^{a}
1.5	45	2.1± 0.32^{a}	3.2 ± 0.26^{d}	48	1.3 ± 0.31^{a}	2.9 ± 3.7^{ab}
2.0	40	1.2 ± 0.56^{a}	0.6 ± 0.32^{a}	45	1.0 ± 0.28^{a}	1.9 ± 0.5^{a}

*Mean ± SD; mean for each experiment marked with the same letter does not differ significantly ($p< 0.05$)

followed by 17.0 shoots in 1.5 mg L^{-1} BAP with 75% shoot regeneration response. The shoot induction and multiple shoot development were observed in all concentrations of BAP (0.1-3.0 mg L^{-1}). Promotion of shoot multiplication by BAP has been reported in other medicinal plants, like *Holostemma ada-kodien*[10], *Macadamia tetraphylla*[11] and *Prunus armenica*[12]. There was significant reduction in multiple shoot induction above 2.0 mg L^{-1} BAP. Similarly fewer shoots were obtained when lower concentrations of BAP (below 1.0 mg L^{-1}) were used. Hence, optimal concentrations of BAP were more efficient to induce multiple shoots. Further, 4-wk-old plantlets (5-7 cm) subcultured on the same medium produced longer shoots (Fig. 1C). Maximum shoot length (15.8 cm) was observed in the presence of 2 mg L^{-1} BAP from nodal buds. Fewer shoots coupled with low frequency of multiple shoot formation were observed when nodal buds were cultured on medium with Kn. Although shooting response (78%) was high, only 3.4 shoots per nodal bud were obtained in 1.0 mg L^{-1} Kn, followed by 2.8 shoots in 0.5 mg L^{-1} of Kn (Table 1).

Shoot tips cultured on MS medium supplemented with growth hormones induced significant multiple shoot development. Maximum number of shoots (10.3) was obtained from explants cultured on MS medium supplemented with 2 mg L^{-1} BAP with 82% shooting response, followed by 9.5 shoots per shoot tip on medium with 1.5 mg L^{-1} BAP with 75% shoot regeneration response (Table 1). Maximum shoot length (7.5 cm) was seen in 2 mg L^{-1} BAP, followed by 4.5 cm in 1.5 mg L^{-1} BAP. Similar results were obtained in *Morus niger*[13] and *Camptotheca accuminata decaisne*[14]. Significant shoot reduction was observed when shoot tips were cultured on medium with Kn. Maximum of 3.3 shoots were obtained per explant on MS medium supplemented with 1.0 mg L^{-1} Kn with 70% shooting response.

In vitro raised multiple shoots were excised into single shoots (7-10 cm) and were rooted on half strength MS medium fortified with various concentrations of auxins. Lower concentrations of auxins (IAA, IBA) enhanced the rooting response (Fig. 1D). Only less number of thin and short rootlets (1.3-3.0) was produced when the shoots were cultured on medium supplemented with various concentrations of IBA or IAA individually (Table 2). However, maximum number of rootlets (14.8) was obtained from the shoots cultured on ½ MS medium augmented with IBA (1.0 mg L^{-1}) and IAA (0.5 mg L^{-1}) together. Thus, synergistic effect of IBA and IAA on the production of rootlets from shoots was seen and thick and significantly long rootlets (11.0 cm) were obtained. Development of such strong and lengthy rootlets would be very much helpful for the establishment of plantlets

Table 2—Rooting response of *in vitro* raised shoots of *B. montanum*

IBA (mg L^{-1})	IAA (mg L^{-1})	Percentage of rooting response	Mean no. of roots per shoot ± S.D*	Mean root length (cm) ± S.D*
0.0	0.5	35	1.6 ± 0.23^{a}	0.5 ± 0.12^{a}
0.0	1.0	53	3.0 ± 1.09^{b}	0.8 ± 0.18^{a}
0.1	0.5	40	2.3 ± 0.71^{b}	2.1 ± 0.26^{b}
0.5	0.1	53	5.6 ± 0.83^{c}	$6.3 \pm 0.43c^{d}$
1.0	0.5	75	7.2 ± 1.81^{d}	8.1 ± 0.56^{d}
1.0	0.5	90	14.8 ± 2.07^{e}	11.0 ± 1.45^{e}
0.5	0.0	32	1.3 ± 0.22^{a}	0.6 ± 0.14^{a}
1.0	0.0	58	2.5 ± 0.54^{b}	4.5 ± 0.39^{c}

* Mean ± SD; mean for each experiment marked with the same letter does not differ significantly ($p < 0.05$)

in the field. Similar results have been reported earlier in *Woodfordia fruiticosa*[15] and *Sterculia urens*[16].

The regenerated plantlets were washed in sterilized water and transferred to plastic cups (10 cm × 8 cm) containing a sterilized mixture of soil and vermiculate (1:1 v/v) and inoculated at 25 ± 2ºC under cool white fluorescent light 70 µmol m^{-2} s^{-1} with a 16-8 h photoperiod. The plastic cover was opened after 15-17 d and temperature was gradually increased to 28 ± 2ºC (Fig. 1E). Out of 110 plantlets transferred to the pots, 86.2 % (95 plants) of the plants got established in natural condition.

The established plants did not show any variation in morphological or growth characteristics when compared to the mother plant. Thus, this efficient *in vitro* procedure of regeneration from nodal bud and shoot tip explants can be used to maintain clonal fidelity of this valuable genotype. This protocol can also be useful for the conservation and mass propagation of this critically endangered medicinal plant.

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