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ARTICLE

In vitro Cloning and Genetic Uniformity Assessment of the Microclones of *Baliospermum Montanum* (Willd.) Muell. Arg.: A Ret Medicinal Species.

Parvathi, K.P.[1], Preetha, T.S.* and Hemanthkumar, A.S.[2]

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Abstract

Baliospermum montanum (Muell) Arg. (Family Euphorbiaceae) is a well known medicinal plant under red list category. The roots, stem, leaves and seeds of this plants are traditionally used for the treatment of different diseases. The phytocomponents viz. 12-deoxy-5βhydrophorbol-13-myristate, 13-palmitate, 12-deoxyphorbol-13-palmitate, baliospermin and montanin were reported in the roots of B. montanum. As because the harvested part is root, the whole plants habitually gets destroyed, which necessitates the need to set up a high frequency in vitro regeneration system for rapid multiplication of this species. The present study reports the regeneration of maximum number of shoots in MS medium supplemented with 1.0 mgl-1 BA and 0.1 mgl-1 NAA. There after a three-fold increase in the rate of shoot multiplication was achieved during subsequent subculture passages in MS medium supplemented with 2.0 mgl-1 BA and 0.1 mgl-1 NAA. A mean number of 3.1±0.17 shoots initiated in 1.0 mgl-1 BA and 0.1 mgl-1 NAA increased to 9.6±0.20 and 18.8±0.20 shoots during first and second subculture passages. The elongated shoots exhibited better rooting response when transferred to ½MS basal as well as the same supplemented with 0.1 mgl-1 NAA. Thus this microcloning protocol offers the production of ~50 plants from a single nodal explant within a period of 3 months. The regenerated plantlets were transferred to the field with 80% success and they grow to maturity without any morphological and genetic abnormalities which is confirmed via ISSR markers thereby offering a suitable in vitro cloning protocol for the supply of physiologically uniform shoot apical meristems for further long-term conservation approaches.

Keywords: *Baliospermum montanum,* Red listed plant, in vitro cloning, genetic uniformity, ISSR markers

Introduction

Baliospermum montanum (Willd.) Muell. Arg. (Family Euphorbiaceae) is a rare, endangered, threatened (RET) medicinal species (FRLHT, 1997) found in Southern Western Ghats of India. Charaka and Sushrutha prescribed this plant for jaundice, anaemia, constipation, disease of abdomen, piles, etc. The roots, stem, leaves and seeds of B. montanum are traditionally used by different tribal communities for the treatment of a variety of ailments. The seeds are externally used as stimulant and rubefacient. The dried roots are anti-helmenthic, diuretic and useful in treating enlarged spleen, and abdominal tumours. Auxillarenic acid is reported in B. montanum seeds; 12-deoxy-5βhydrophorbol-13-myristate, 13-palmitate, 12-deoxyphorbol-13-palmitate, baliospermin and montanin are present in the roots (Rastogi and Mehrotra, 1993). Pharmacological screening of the plant has given away its antibacterial, hepatoprotective, anticancerous, free radical scavenging, immuno-modulatory and anthelmintic potentialities (Mali and Wadekar, 2008).

The plants of B. montanum is propagated conventionally by seeds and vegetative cutting. However, the non-availability of seeds due to high sterility and requirement of large amount of planting materials are major drawbacks of conventional propagation. Due to injudicious overexploitation from the natural habitat and the lack of appropriate cultivation practices, this important medicinal species is found diminishing from the wild and there is an immediate need to conserve this species using in situ and ex situ methods.

There are few studies on in vitro propagation of B. montanum (Johnson and Manickam, 2003; Satheesh George et al., 2008; Sasikumar et al., 2009); so far there is no report on in vitro conservation strategies especially cryopreservation, for this Red listed medicinal plant for which in vitro shoot culture establishment is a prerequisite for providing physiologically uniform explants (shoot tips) for cryopreservation. Hence the study presented here describes a reliable rapid high frequency clonal multiplication of B. montanum and the genetic uniformity assessment of the regenerated plants using ISSR markers.

[1]Plant Tissue Culture Laboratory, Department of Botany, University College, Thiruvananthapuram 695034, Kerala, India
[2]Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram 695562, Kerala, India
*Corresponding author, e mail: preethahemanth@yahoo.com

Materials and Methods

Herbal material and preparation of extract
Explant Collection and Sterilization

Baliospermum montanum (Willd.) Muell. Arg. plants growing from the natural habitat (Peringamala-Palode, Thiruvananthapuram, Kerala, India) were collected and grown in the green house of Department of Botany, University College, Thiruvananthapuram, Kerala, India. The explants collected from these field-grown plants were used for in vitro cloning experiments.

Shoot Culture Establishment

Nodal segments (first, second, third and fourth nodes) of B. montanum isolated from the greenhouse-maintained plants were thoroughly washed under running tap water and subsequently in 5% (v/v) labolene, a liquid detergent for 20-30 minutes and washing again in running tap water and rinsing in distilled water. They were surface sterilized with 0.1% (w/v) HgCl2 for 8 minutes and subsequently rinsed in sterile distilled water. The explants excised aseptically were inoculated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.5, 1.0 and 2.0 mgl-1 BA/ Kinetin/2-iP either individually or 0.5, 1.0 and 2.0 mgl-1 BA in combination with 0.5 mgl-1 Kinetin or 0.1 mgl-1 NAA/ IAA and incubated under the standard culture conditions (26± 2oC) under 12 hour photoperiod at a photon flux intensity of 50-60 µEm-2s-1 provided by cool, white, fluorescent tubes (Philips, India) with 50-60 % RH. The shoots developed in the initiation medium were subcultured to fresh medium of the same composition for multiple shoot induction for 3-4 subcuture passages at an interval of 4 weeks period.

Rooting, Hardening and Field Transfer

Shoots elongated (5-7 cm) during the shoot multiplication step were transferred to ½MS medium devoid of growth regulators or supplemented with 0.1 mgl-1 NAA for the induction of roots. The rooted plantlets were washed thoroughly in running tap water, treated with fungicide and transplanted in polybags filled with potting medium of garden soil and river sand mixture (1:1) and hardened by covering them with polythene bags for initial ten days. The established plantlets during hardening were then transferred to the field to facilitate their establishment.

Genetic Uniformity Analysis Using ISSR Markers

Randomly selected leaf samples from eight field-grown plants were used for genetic fidelity analysis. Total genomic DNA from the samples was isolated using cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). After ethanol precipitation DNA was resuspended in 100 µL 1xTE buffer (pH 8.0). ISSR assay was carried out in 25 µL reaction mixture containing 0.2 mMdNTP's, 10 mMTris-HCL, 1.5 mM MgCl2, 50 mMKCl, 0.1% Triton X-100, 1.0 U Taq DNA polymerase (Finzymes, Helsinki, Finland), 15 pmol primers (IDT, Coralville, USA) and 50 ng of genomic DNA. The amplification was performed in a thermal cycler (Eppendorf ESP-S). At an initial cycle of 2 minutes at 93 °C, 2 min at 50 to 55 °C, and 2 minutes at 72 °C. A total of 39 cycles of 1 minute at 93 °C, 1 minute at 50 to 55 °C and 1 minute at 72 °C were performed. The last cycle was performed by 10 minutes extension at 72 °C. Reaction mixture wherein template DNA replaced by distilled water was used as negative control. Amplified products where resolved in 1.4% agarose gel (1xTBE) followed by EtBr staining. Amplification with each arbitrary primer was repeated 3 times and those primers thatproduced reproducible and consistent bands were selected for data generation.

Statistical Analysis

The data on surface sterilization, shoot initiation and shoot multiplication over two subculture passages were recorded at regular intervals. It was statistically analysed using ANOVA and the means were compared using t-test at $p \leq 0.05$.

Results and Discussion

Surface Sterilization

Considering the preservation of biodiversity of plant genetic resources, conservation of medicinal plant species through in situ and ex situ approaches are taken for addressing the issues of their diminished supply and gene erosion. Depending on the biological nature of the species to be conserved, different ex situ conservation strategies like seed banking, field gene bank, in vitro and cryobanking are adopted generally. Preservation of plant genetic resources in field collections is risky, as the valuable germplasm can be lost because of the attack of pests, diseases and adverse unpredicted weather conditions. Also, the upholding of clonal orchards in land is not economical and it is labour-intensive too. In this context, utilizing the biotechnological approaches as ex situ conservation programmes becomes crucial. Incorporation of tools like in vitro culture, cryopreservation and molecular markers in plant germplasm conservation is a prerequisite to achieve success in sustainable utility and to supplement the existing ex situ conservation methods. This offers a valuable alternative to plant diversity studies, management of genetic resources and ultimately conservation of plant biodiversity. The in vitro conservation consist of meristem-based micropropagation, somatic embryogenesis, cell culture technologies and embryo rescue techniques, as well as a range of in vitro cold storage and cryopreservation protocols. Several in vitro techniques have been developed for preserving vegetatively propagated and recalcitrant seed producing species (Berjak et al., 2010; Engelmann, 2011). Germplasm conservation and rapid mass propagation of medicinal plants also can be achieved through in vitro techniques and cryopreservation and has been successfully extended to propagate endangered species (Keshavachandran et al., 2005).

The plants growing in the natural habitat are invariably associated with several microorganisms and therefore sur-

Table 1. Standardization of $HgCl_2$ exposure period during surface sterilization in different explants of *B. montanum*

Explant type	$HgCl_2$ exposure period (minutes)	Response %*
Shoot tips	5	23.33[f]
	7	43.33[d]
	9	76.67[b]
	10	76.67[b]
Node (I)	5	20.00[f]
	7	46.67[d]
	9	76.67[b]
	10	80.00[a]
Node (II)	5	20.00[f]
	7	36.67[e]
	9	46.67[d]
	10	53.33[c]
Node (III)	5	16.67[g]
	7	33.33[e]
	9	43.33[d]
	10	43.33[d]
Node (IV)	5	10.00[g]
	7	23.33[f]
	9	36.67[e]
	10	33.33[e]

Data represents mean values of ten replicates repeated thrice, recorded after 4 weeks of culture. The mean values followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and t-test at p≤0.05

face decontamination of the explants is an inevitable process to initiate an aseptic in vitro culture system. Several types of aseptic sterilization processes and surface sterilants were used for this purpose. As a general procedure, the explants were initially cleaned with a detergent solution and then washed thoroughly in running tap water. Further processing such as selection of surface sterilant and duration of treatments solely depends on the type/ maturity of explants/ tissue. Surface sterilization may be achieved with either single or successive use of more than one surface sterilant. The surface decontamination initially with 5% (v/v) labolene followed by aseptic treatment with 0.1% (w/v) HgCl2 for 8-10 minutes offered a reasonable decontamination protocol for B. montanum in the present study.

Shoot tip and nodal segments from first, second, third and fourth nodes collected during March-April season used as the explant variants for clonal multiplication, after surface sterilization in 0.1% HgCl2 for 5-10 minutes when inoculated in MS medium supplemented with 0.5 mgl-1 BA exhibited different percentage responses upon different HgCl2 exposure periods. Maximum 76.67% explants responded in the case of shoot tips after 9-10 minutes treatment (Table 1). Nodal segments from first node showed 80% response after 10 minutes of HgCl2 treatment, which was the highest response percentage among all the explants types tested. Second, third and fourth nodal explants even after 10 minutes HgCl2 treatment exhibited very little percentage of infection-free cultures (53.33, 43.33 and 33.33% respectively) (Table 1). As the first nodal explants showed better shoot induction and shoot multiplication efficiency than shoot tips, they were selected as the suitable explants for in vitro clonal multiplication of B. montanum. In the present study the percentage of infection-free cultures were maximum (80%) when first nodal segments were used as explants. The morphogenic frequency of shoot bud induction and shoot multiplication recorded was higher in nodal segments when compared to shoot tips as observed in earlier studies (Ong Poh Liang and Chan Lai Keng, 2006; Sasikumar et al., 2009).

Shoot Culture Establishment

Shoot tip and nodal segments collected during March-April season used as the explant after the surface sterilization procedure described earlier when cultured on MS basal medium supplemented with different plant growth regulators produced more than one shoot in all the treatments. Significant differences were observed in number of shoots per explant among different concentrations of plant growth regulators during culture initiation as well as multiplication. All the concentrations of BA and Kinetin facilitated the differentiation of shoot buds. In MS medium supplemented with 2.0 mgl-1 BA, a mean number of 3.1±0.26 shoots were produced (Fig. 1a) (Table 2). Combination of the two cytokinins viz. BA and Kinetin effected multiple shoot bud induction in B. montanum compared to that of either BA alone. A linear increase in mean number and mean length of shoots were noticed with increase in concentration of BA in combination with Kinetin. Combination of 0.5 mgl-1 Kinetin and 0.5, 1.0 and 2.0 mgl-1 BA produced a mean number of 1.4±0.20, 1.8±0.26 and 2.8±0.26 shoots per explant respectively (Fig. 2b) (Table 2). Thus the frequency of multiple shoot induction increased when BA was used in combination with Kinetin. However, the explants showed maximum rate of shoot multiplication and the shoots were comparatively longer among the combinations of BA and NAA/ IAA. MS medium supplemented with 2.0 mgl-1 BA and 0.1 mgl-1 NAA/ IAA showed better results in terms of shoot multiplication with a mean number of 3.6±0.20 and 3.0±0.30 shoots per explants respectively (Fig. 1c) (Table 2). This hormonal combination was found to be the best

Table 2. Morphogenic response of *B. montanum* during shoot culture initiation

Plant growth regulators (mgl^{-1})					% response	MSN	MSL (cm)
BA	Kinetin	2-iP	NAA	IAA			
0.5	-	-	-	-	76.67[b]	1.0±0.17[c]	0.75±0.08[c]
1.0	-	-	-	-	70.00[b]	1.3±0.15[c]	0.91±0.06[c]
2.0	-	-	-	-	80.00[a]	1.5±0.16[c]	0.93±0.04[c]
-	0.5	-	-	-	73.33[b]	1.2±0.13[c]	0.76±0.04[c]
-	1.0	-	--	-	73.33[b]	1.1±0.09[c]	0.89±0.03[c]
-	2.0	-	-	-	76.67[b]	1.1±0.09[c]	0.92±0.04[c]
-	-	0.5	-	-	75.05[b]	1.0±0.03[c]	4.0±0.04[a]
-	-	1.0	-	-	72.07[b]	1.2±0.64[c]	4.4±0.16[a]
-	-	2.0	-	-	78.23[b]	1.8±0.34[c]	4.8±0.26[a]
0.5	0.5	-	-	-	70.00[b]	1.5±0.16[c]	1.25±0.08[b]
1.0	0.5	-	-	-	83.33[a]	1.9±0.26[c]	1.35±0.05[b]
2.0	0.5	-	-	-	80.00[a]	2.0±0.20[b]	1.37±0.04[b]
0.5	-	-	0.1	-	76.67[b]	2.4±0.16[b]	1.60±0.06[b]
1.0	-	-	0.1	-	76.67[b]	3.1±0.17[a]	1.78±0.06[b]
2.0	-	-	0.1	-	80.00[a]	2.9±0.17[b]	1.84±0.03[b]
0.5	-	-	-	0.1	70.00[b]	2.6±0.14[b]	1.63±0.07[b]
1.0	-	-	-	0.1	73.33[b]	3.0±0.12[a]	1.82±0.04[b]
2.0	-	-	-	0.1	76.67[b]	2.8±0.24[b]	1.76±0.03[b]

MSN Mean shoot number; MSL Mean shoot length

Data represents mean values ± SE of 10 replicates repeated thrice, recorded after 4 weeks of culture. The mean values followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and t-test at p≤0.05.

among the various combinations tested.

Shoot Multiplication during Subculture Passages

A three-fold increase in the rate of shoot multiplication was noticed in shoot cultures during first subculture passages in MS medium supplemented with 0.5-2 mg/l 2-iP, 1.0-2.0 mgl-1 BA in combination with 0.1 mgl-1 NAA/ IAA, where proliferation of axillary buds of individual shoots was noticed. The rate of shoot multiplication increased in shoot cultures in MS medium supplemented with 0.5-2.0 mgl-1 2-iP where proliferation of axillary buds of individual shoots were also resulted. A mean number of 3.1±0.17 shoots initiated in 1.0 mgl-1 BA and 0.1 mgl-1 NAA increased to 9.6±0.20 and 18.8±0.20 shoots respectively during first and second subculture passages (Table 3). Similar trend in the multiplication rate was observed in the present study with regard to all the plant growth regulator treatments. A 10-fold increase (10.67 and 10.23) in the multiplication rate was recorded after second subculture passage in the treatment combination of 1.0 and 2.0 mgl-1 BA and 0.1 mgl-1 IAA (Table 3). Once aseptic shoots were induced, the successive multiplication rate gets maximized under in vitro conditions in many plant species (Bhoumik et al., 2009; Preetha et al., 2014) which may be due to the triggering of

Table 3. Scaling up of shoot multiplication in *B. montanum* during subculture passages

Plant growth regulators (mgl^{-1})					MSN during shoot initiation	MSN during 1st subculture	Fold increase after 1st subculture	MSN during 2nd subculture	Fold increase after 2nd subculture
BA	Kinetin	2-iP	NAA	IAA					
2.0	-	-	-	-	1.5±0.16^{c}	3.1±0.26^{e}	2.07	8.4±0.29^{e}	5.60
2.0	0.5	-	-	-	2.0±0.20^{b}	6.8±0.26^{b}	3.40	13.0±0.36^{c}	6.50
1.0	-	-	0.1	-	3.1±0.17^{a}	9.6±0.20^{a}	3.10	18.8±0.20^{a}	6.06
2.0	-	-	0.1	-	2.9±0.17^{b}	9.2±0.28^{a}	3.17	15.2±0.28^{a}	5.24
-	-	0.5	-	-	1.0±0.03^{c}	2.2±0.24^{f}	2.20	4.5±0.56^{h}	4.50
-	-	1.0	-	-	1.2±0.64^{c}	2.8±0.36^{f}	2.33	5.2±0.08^{g}	4.33
-	-	2.0	-	-	1.8±0.34^{c}	3.4±0.08^{e}	1.89	6.0±0.88^{f}	3.33
1.0	-	-	-	0.1	1.2±0.14^{c}	5.5±0.20^{c}	4.58	12.8±0.33^{d}	10.67
2.0	-	-	-	0.1	1.7±0.20^{c}	4.0±0.30^{d}	2.35	17.4±0.29^{b}	10.23

MSN Mean Shoot number; Data represents mean values ± SE of 10 replicates repeated thrice, recorded after 4 weeks of culture. The mean values followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and t-test at p≤0.05.

accelerated axillary meristem proliferation upon the effect of plant growth regulator treatments.

Shooting Elongation, Rooting, Hardening and Field Transfer

The shoots initiated during culture initiation elongated substantially within 4-6 weeks. The elongated shoots showed better rooting response when transferred to ½MS basal as well as medium supplemented with 0.1 mgl-1 NAA (Fig. 1d). This observation is in contrast with the published micropropagation reports in the plant, wherein full strength MS medium supplemented with IAA/ IBA induced effective rooting compared to NAA (Johnson and Manickam, 2003; Singh et al., 2003; Satheesh George et al., 2008).

The plantlets raised were transferred to river sand and soil mixture (1:1) in polybagss and kept inside polythene covering. They were hardened for 4 weeks period which exhibited 90% survival rate (Fig.1f-h). Finally they were transferred to the field were they got established at 80% rate. The plants showed signs of growth with the emergence of a new leaves in two weeks thereby establishing a reliable hardening and nursery management package for large scale cultivation, conservation and consistent utilization of this medicinal resource.

Beginning the aseptic procedures with a single node as explant, 3 shoots proliferated during shoot culture initiation was multiplied to 9.6 in the first subculture and to 18.8 in the second subculture passage. Thus it is possible to produce approximately 50 plants (1 node → 3 in vitro shoots; each of them proliferates into 9.6 in the first subculture and to 18.8 in the second subculture passages. Hence the total number of plantlets produced per an explant is 18.8 × 3 = 56.4. Among which, approximately 50 plantlets will be survived after hardening and field transfer) within a period of 12 weeks time.

Genetic Uniformity analysis using ISSR Markers

The plantlets transferred to field were found to be genetically uniform and exhibited monomorphic bands with ISSR primers (Fig.1i). Genetic fidelity of acclimatized plants assessed by using inter-simple sequence repeat analysis showed genetic stability in agreement with the finding in Phyllanthus fraternus (Upadhyay et al., 2015).

Conclusion

The study has standardized a simple and viable in vitro clonal multiplication of B. montanum and by the genetic uniformity of the microclones was confirmed by ISSR markers. The protocol can be extended for the isolation of shoot apical meristems for cryopreservation of this Red listed medicinal plant thereby ensuring the long-term conservation of this high sought medicinal species.

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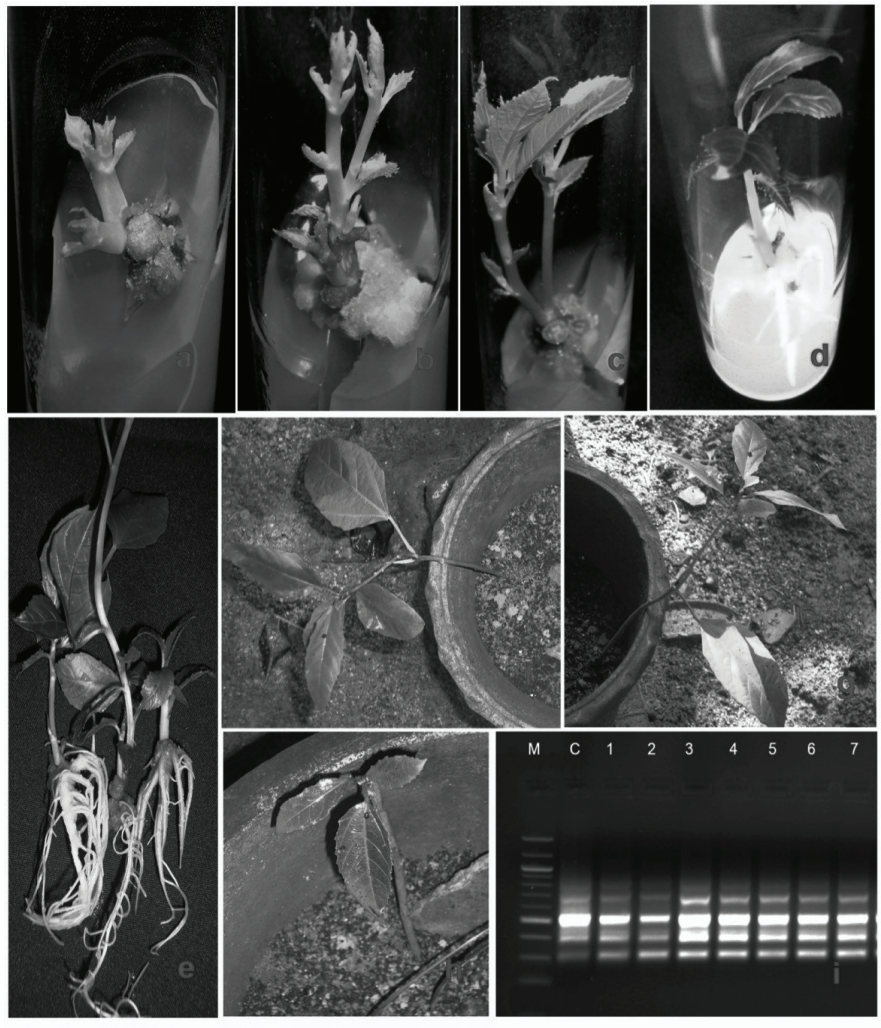

Figure 1(a-h)- In vitro cloning of *B. montanum*: a. Initiation of multiple shoots along with basal friable callus formation from nodal explants in MS+2.0 mgl-1 BA after 4 weeks, b. Shoot elongation along with multiplication in MS+2.0 mgl-1 BA+0.5 mgl-1 Kinetin after 4 weeks; c. Shoot elongation along with multiplication in MS+2.0 mgl-1 BA+0.1 mgl-1 NAA after 4 weeks; d. Rooting of shoots in ½MS+0.1 mgl-1 NAA after 3 weeks; e. Rooted plantlets; f-h. Established in vitro-raised plantlets of *B. montanum*; i. ISSR banding pattern of in vitro-derived plantlets.

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