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RESEARCH PAPER

High frequency *in vitro* regeneration system for conservation of *Barleria prionitis* L., a threatened medicinal shrub

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ABSTRACT

A rapid and efficient plant propagation system through nodal and embryo cultures was developed for conservation of threatened shrub *Barleria prionitis*. Nodal explants exhibited high frequency shoot proliferation on Murashige and Skoog (MS) medium supplemented with 1.0 mg l^{-1} 6-benzylaminopurine (BA) and 0.5 mg l^{-1} thidiazuron (TDZ). Microshoots were best rooted on half-strength MS fortified with 0.5 mg l^{-1} indole-3-butyric acid (IBA). Maximum conversion (63.6 %) of zygotic embryos into well rooted plantlets was achieved on half-strength MS supplemented with 20 mg l^{-1} sucrose, devoid of any growth regulator S. Plantlets with high PSII photochemical efficiency (Fv /Fm ≥ 0.8) were successfully shifted to natural conditions. The overall survival rate during acclimatization from in vitro growth to field transfer was 81 %. The developed micropropagation protocol can be successfully used for large-scale multiplication and conservation this high value medicinal plant species.

Keywords: micropropagation; embryo culture; *in vitro* rhizogenesis; chlorophyll fluorescence; acclimatization

РЕЗЮМЕ

Сони В., Кумари Р., Шварнкар П.Л. Система интенсивной регенерации *in vitro* для сохранения *Barleria prionitis* L., редкого лекарственного кустарника. Разработана быстрая и эффективная система размножения растений через меристемные и эмбриональные культуры для сохранения угрожаемого кустарникового вида *Barleria prionitis*. Меристемные эксплантаты демонстрировали интенсивную пролиферацию побегов на среде Murashige и Skoog (MS), дополненную 1,0 мг/л 6-бензиламинопурином (BA) и 0,5 мг/л тидиазуроном (TDZ). Микропобеги лучше всего укоренялись на MS, обогащенной 0,5 мг/л индол-3-масляной кислотой (IBA). Максимальное преобразование (63,6 %) зиготных эмбрионов в укоренившиеся растения было достигнуто на MS, дополненной 20 мг/л сахарозы, лишенной какого-либо регулятора роста S. Растеньица с высокой фотохимической эффективностью PSII (Fv / Fm ≥ 0,8) были успешно перенесены в естественную среду. Общая выживаемость при акклиматизации от роста *in vitro* до переноса в естественную среду составляла 81 %. Разработанный протокол микроразмножения может быть успешно использован для крупномасштабного размножения и сохранения этих высокоценных лекарственных растений.

Ключевые слова: микроразмножение, культура эмбрионов, корнеобразование *in vitro,* флуоресценция хлорофилла, акклиматизация

Barleria prionitis L., (Acanthaceae), an annual medicinal shrub of arid and semiarid regions of Africa and Asia, is used in the Indian and Chinese traditional system of medicine since prehistoric time. It have shown antiviral (Chen et al. 1998), anti-spermatogenic (Verma et al. 2005), hepatoprotective (Singh et al. 2005), antidiabetic (Dheer & Bhatnagar 2010), anti-nociceptive (Jaiswal et al. 2010), anti-inflammatory (Khadse & Kakde 2011), antifungal (Amoo et al. 2011), diuretic (Musale et al. 2011), antibacterial (Diwan & Gadhikar 2012), antidepresent (Gangophadhyay et al. 2012), immunomodulatory (Ghule & Yeole 2012), anti-arthritic (Choudhary et al. 2014) and antifertility (Singh & Gupta 2016) activities. The plant is especially well known for treating bleeding gums and toothache (Gupta et al. 2016). Unfortunately, the wild population of this plant is now threatened into extinction because of over exploitation and lack of organized cultivation (Walter & Gillett 1998). Conventionally *B. prionitis* is propagated mainly through the seeds and stem cuttings. However, germination of seeds is poor and propagation through stem cuttings solely relies on season for multiplication, which makes it an inefficient way for the conservation of this medicinally important plant. So this plant species requires immediate attention for its protection, large scale systematic cultivation and conservation.

Plant tissue culture is an alternative to the conventional methods of propagation with the objective of enhancing the rate of multiplication and conserving the threatened medicinal plant species. The large scale production of micropropagated plantlets is often limited by poor survival when plantlets are transferred in *ex vitro* conditions. The high exogenous sucrose content in the medium has been shown to suppress photosynthetic gene expression, reduce chlorophyll content, reduce Calvin cycle enzymes, as well as reduce Rubisco activity and Rubisco concentration, leading to low photosynthetic rates (Van Huylenbroeck & Debergh 1996, Sinha et al. 2002, Fuentes et al. 2005). The benefit of any micropropagation system can, however, only be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found *ex vitro*.

The establishment of tissue culture protocol will be an important action for multiplication and germplasm conservation of *B. prionitis*. Thus, the objective of this work was to develop a highly efficient *in vitro* regeneration protocol of *B. prionitis* through nodal and embryo cultures. The present work also evaluated effects of altering medium strength and sucrose concentration on in vitro zygotic embryo germination. In addition, assessment of photosynthetic performance of hardened plants by chlorophyll a fluorescence analysis was carried out to ensure their the high survival rate in natural conditions.

MATERIAL AND METHODS

Plant material and surface sterilization

Nodal explants and seeds were collected from healthy plants of *B. prionitis* growing in the Jhanana Nursery, Jaipur (India). The excised nodal explants were washed thoroughly under running tap water for 30 min to eliminate dust particles and then with 5 % teepol for 8–10 min and rinsed several times in sterile distilled water. Then, the explants were treated with an antifungal agent (Bavistin) for 1 hour and the again rinsed three times with sterile distilled water. Thereafter, the explants were surface sterilized under a laminar flow chamber with aqueous solution of 0.1 % $HgCl_2$ for 2 min and finally washed with sterile distilled water for 2–3 times. Similarly, the seeds were surface disinfected and zygotic embryos were aseptically excised by cracking the seed coats.

Culture media and growth conditions

The sterilized nodal explants were cultured on MS Medium (Murashige & Skoog 1962) supplemented with 3 % (w/v) sucrose and various combinations/concentrations of plant growth regulators. The zygotic embryos were inoculated at full or half strength MS supplemented with various concentrations sucrose (10, 20 and 30 g l^{-1}). The pH of the media was adjusted 5.8 before autoclaving 121°C for 15 min. All the cultures were maintained at 25±2°C and 65–70 % relative humidity with photoperiod of 16-h using a photosynthetic photon flux density (PPFD) of 40 mmol m^2 s^{-1} provided by cool white fluorescent tubes (Philips, India).

In vitro rhizogenesis and hardening

Elongated shoots with 2–3 pairs of healthy leaves were excised and transferred to rooting medium. The shoots were cultured on MS supplemented with various auxins viz. IBA, IAA and NAA. Well rooted plantlets, derived from both nodal and embryo cultures, were gently washed in sterile water and transferred to plastic cups (10 × 8 cm) containing sterilized mixture of sterile soil, sand and coco peat (1 : 2 : 1). The plantlets covered in transparent polyethylene bags were kept for 4 weeks in growth chamber at 25 ± 2°C with 16 h photoperiod and 40 mmol m^2 s^{-1} of irradiation. The plantlets were irrigated with tap water. The irrigation schedule and volume of water was calibrated to keep the pot mixture saturated and prevent flooding. The hardened plants were subsequently transferred to large pots containing normal garden soil and were maintained in an open greenhouse without environmental conditioning for 4 weeks.

Measurement of Fv /Fm and acclimatization

Photosynthetic screening during hardening process can help to improve the performance and survival of micropropagated plants. Thus, maximum quantum yield of primary photochemistry $\varphi P_0 = TR_0/ABS = (Fm-F_0)/Fm = Fv/Fm$ (where TR and ABS denote the trapped and absorbed excitation energy fluxes) of plantlets growing under growth chamber and green house conditions was regularly measured using a Plant Efficiency Analyser, PEA (Hansatech Instruments, Kings Lynn, Norfolk, U.K.) according to Heber et al. (2011). The leaf samples were dark adapted for 2 hours before the fluorescence measurements. Plantlets having high PSII photochemical efficiency (Fv /Fm ≥ 0.8) were shifted to natural conditions. The survival rate of plantlets was recorded after 1 month of transfer to natural conditions.

Experimental design and statistical analysis

All the experiments were conducted with a minimum of 30 replicates per treatment and each experiment was repeated thrice. The data were analyzed statistically by one-way analysis of variance (ANOVA) followed by Tukey's test at P = 5 % using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA) and data represented as mean ± standard error (SE).

RESULTS AND DISCUSSION

In vitro establishment and multiplication

Development of single unhealthy (yellowish) shoot followed by the formation of green nodular callus at the basal part of nodal explants was observed at 0.5 mg l^{-1} BA after 9 days of culture (Fig. 1 A, B). TDZ alone in different concentrations (0.5, 1.0, 2.5 mg l^{-1}) failed to induce high frequency shoot bud proliferation in vitro. Development of healthy shoot buds was recorded when low concentration of TDZ (0.5 mg l^{-1}) was incorporated in MS along with BA (Table 1). The best response in terms of explants plant response (%) and mean shoot length was observed on MS supplemented with 1.0 mg l^{-1} BA and 0.5 mg l^{-1} TDZ (Fig. 1 C).

Frequency of shoot induction was drastically decreased with increasing concentration of BAP, either singly or in combination with TDZ (0.5 mg l^{-1}). An inhibitory effect of higher concentrations of BA on in vitro shoot proliferation has also been reported earlier in *Albizia chinensis* (Osbeck) Merr. (Sinha et al. 2000), *Pterocarpus marsupium* (Swynn. ex Baker f.) Swynn. ex Steedman (Anis et al. 2005), *Arachis hypogaea* L. (Banerjee et al. 2007), *Doritis pulcherrima* Lindl. (Mondal et al. 2013) and *Salvia splendens* Sellow ex Schult.

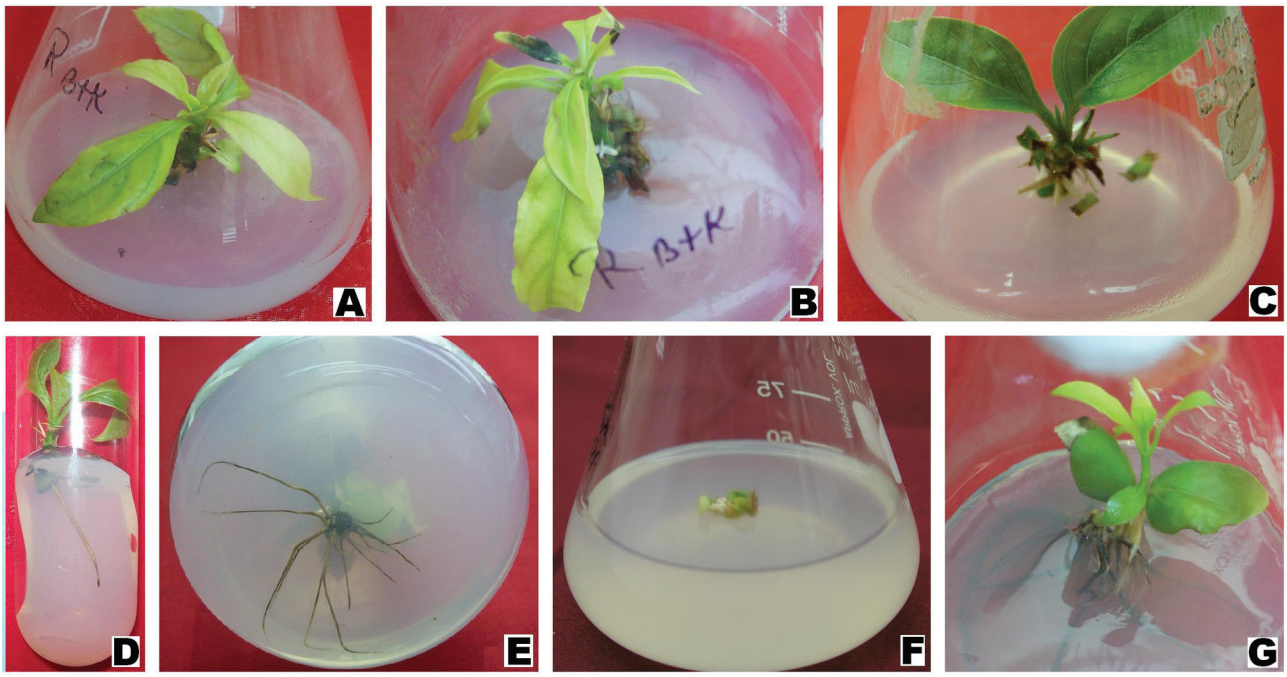

Figure 1 *In vitro* propagation of *B. prionitis*. A, B – shoot induction from nodal explant on MS with 0.5 mg l^{-1} BA, and (C) 1.0 mg l^{-1} BA and 0.5 mg l^{-1} TDZ, 4-week-old culture. D – *in vitro* rhizogenesis on half-strength MS with 2.0 mg l^{-1} IBA, and (E) 0.05 mg l^{-1} IBA, 4-week-old culture. F – *in vitro* germination of zygotic embryo, and G – well rooted plantlet developed from zygotic embryo culture on half-strength MS with 20 mg l^{-1} sucrose.

(Sharma et al. 2014). Other plant growth regulators, singly or in combinations could not initiate any significant morphogenetic response in nodal explants of *B. prionitis*.

In vitro rhizogenesis

Overall, MS of half-strength proved better for root induction as compared to full strength MS whether with or without IBA of any concentration (Table 2). The rooting characters like percentage of shoots producing roots, mean no. of root/shoot and mean root length (cm) have been influenced by the MS strength and IBA concentration. Half-strength MS along with higher concentration of IBA could induce rhizogenesis in vitro at low frequency (Fig. I D). Medium having half-strength MS supplemented with 0.5 mg l^{-1} IBA recorded to be the best root inducing medium for microshoots developed from nodal explants of *B. prionitis* (Fig 1 E). Similarly, the effect of IBA on root induction has been reported in many plant species, i.e. *Plectranthus bourneae* Gamble (Thaniarasu et al. 2015), *Passiflora foetida* L. (Shekhawat et al. 2015), *Morinda coreia* Buch.-Ham. (Shekhawat et al. 2015), *Ceropegia evansii* McCann (Chavan et al. 2015).

Embryo germination

Carbohydrates play an important role in the maintenance of an adequate osmotic balance in the growth medium as well as in the promotion of embryo growth. Sucrose has been widely used as an energy source because explants cultivated in vitro are heterotrophic and depend on external energy and carbon sources (Hu & Ferreira 1998). Sucrose concentration is important in early stages of zygotic embryo germination as young embryos require a complex medium for growth and maturation. In present study, a highly significant effect of altering medium strength and sucrose concentration was observed on germination of zygotic embryos of *B. prionitis* (Table 3). Considering all of the variables examined (percent germination, mean plant height and mean no. of root/plant), half-strength MS fortified with 20 mg l^{-1}sucrose, devoid of any growth regulators, was the best culture medium for obtaining in vitro differentiation of zygotic embryos into plantlets (Fig 1 F, G). Higher concentration of sucrose (30, 40 mg l^{-1}) showed an inhibitory effect on embryo germination.

Hardening and acclimatization

The increased use and efficiency of micropropagation is still restricted by the high percentage of plants which are lost or damaged when transferred to *ex vitro* conditions (Pospíšilová et al. 1999). It is due to a poor photosynthetic capacity of in vitro-cultured plantlets, apparently caused by insufficient inflow of carbon dioxide and the sucrose added to growth medium causing negative feedback for photosynthesis. Therefore, screening photosynthetic/autotrophic potential of hardened plants is essential to optimize hardening duration to ensure their high survival rate in ex vitro conditions. In the present investigations, plantlets regenerated in vitro were successfully acclimatized in the growth chamber (88 % survival) and then in the greenhouse (92 % survival). The Fv/Fm value, a sensitive and early indicator of photo-inhibition and changes in photochemical efficiency, increased throughout hardening process, providing evidence for increasing photosynthetic performance. *In vitro* developed plants of *B. prionitis* significantly achieved Fv / Fm value from 2.34 to 8.06 within 6 weeks of hardening process in growth chamber and green house conditions (Fig 2 A).

Table 1. Influence of BA and TDZ on shoot proliferation and regeneration after 4 weeks of culture of nodal explants of *B. prionitis*. Values represent the mean of 30 replicates ± standard error (SE).

Plant growth regulators (mg l^{-1})		Explant response (%)	Mean shoot length (cm)
BA	TDZ		
0.5	0.0	23.0 ± 2.1	0.3 ± 0.03
1.0	0.0	17.7 ± 3.3	0.9 ± 0.04
2.5	0.0	12.6 ± 2.2	0.6 ± 0.02
5.0	0.0	9.6 ± 1.7	0.4 ± 0.01
0.0	0.5	21.9 ± 2.1	0.5 ± 0.06
0.0	1.0	16.6 ± 2.0	0.6 ± 0.07
0.0	2.5	12.4 ± 2.3	0.7 ± 0.10
0.5	0.5	56.6 ± 2.7	0.9 ± 0.04
1.0	0.5	87.2 ± 3.6	2.5 ± 0.10
2.5	0.5	38.4 ± 3.1	0.8 ± 0.06
5.0	0.5	18.5 ± 2.0	0.7 ± 0.04
0.5	1.0	22.8 ± 2.9	0.9 ± 0.07
1.0	1.0	9.7 ± 2.2	0.6 ± 0.04
0.5	2.5	14.9 ± 3.3	0.6 ± 0.01
1.0	2.5	5.4 ± 2.1	0.4 ± 0.10

Table 2. Influence of IBA on *in vitro* rhizogenesis in *B. prionitis* after 4 weeks of culture. Values represent the mean of 30 replicates ± standard error (SE).

Media Combinations	% Rooting	Mean no. of root/ shoot	Mean root length (cm)
MS full strength	-	-	-
MS full strength + 0.5 mg l^{-1} IBA	22 ± 2.2	1.14 ± 0.3	0.77 ± 0.2
MS full strength + 1.0 mg l^{-1} IBA	34 ± 4.1	1.79 ± 0.5	2.26 ± 0.7
MS full strength + 2.0 mg l^{-1} IBA	36 ± 4.4	2.74 ± 0.4	2.29 ± 0.4
MS half strength	56 ± 2.6	1.58 ± 0.3	2.61 ± 0.8
MS half strength + 0.5 mg l^{-1} IBA	94 ± 6.8	6.50 ± 1.1	3.78 ± 0.3
MS half strength + 1.0 mg l^{-1} IBA	64 ± 4.3	8.50 ± 1.4	4.13 ± 0.5
MS half strength + 2.0 mg l^{-1} IBA	53 ± 6.6	2.66 ± 0.5	1.11 ± 0.4

Table 3. Influence of medium strength and sucrose concentration on embryo germination. Values represent the mean of 30 replicates ± standard error (SE).

Medium strength	Sucrose (mg l^{-1})	Germination (%)	Mean plant Height (cm)	Mean no. of root/plant
Half	0	26 ± 3.1	1.5 ± 0.2	1.5 ± 0.6
	10	56 ± 4.5	2.3 ± 0.5	2.3 ± 0.7
	20	94 ± 3.4	3.6 ± 0.7	8.5 ± 1.1
	30	81 ± 5.1	1.6 ± 0.3	4.4 ± 0.5
	40	65 ± 4.2	1.2 ± 0.3	4.2 ± 0.3
Full	0	12 ± 2.1	1.3 ± 0.3	1.0 ± 0.4
	10	18 ± 2.4	1.2 ± 0.3	1.5 ± 0.2
	20	38 ± 1.8	2.3 ± 0.4	2.0 ± 0.7
	30	26 ± 3.2	1.5 ± 0.2	2.1 ± 0.4
	40	9 ± 2.1	1.3 ± 0.5	1.4 ± 0.3

Fully hardened plants of about 8 cm having high PSII photochemical efficiency (Fv/Fm ≥ 0.8) were shifted to plastic bags containing garden soil and maintained under natural conditions with 100 % survival rate (Fig. 2 B). The developed in vitro protocol can be useful for the conservation and mass propagation of this threatened medicinal plant species. Photosynthetic screening of in vitro raised plantlets, as described in present study, could also be used for the acclimatization of other threatened species with high survival rate and their eventual establishment in the field.

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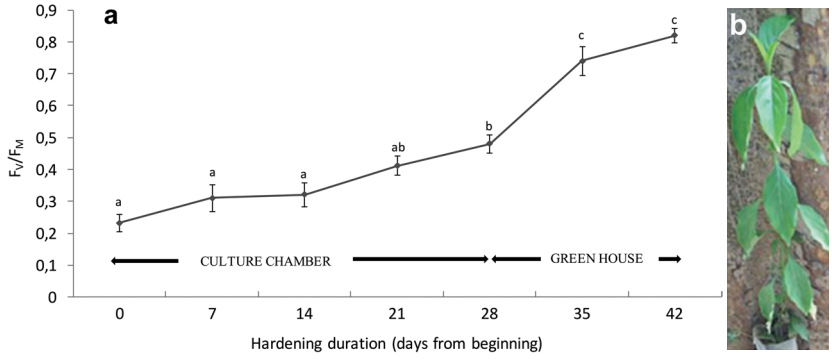

Figure 2. a – Fv/Fm (TR0/ABS) values of *in vitro* raised plantlets during hardening process in growth chamber and green house (Bars represent the mean of 30 replicates ± standard error (SE). Mean values indicated by the same superscript are not significantly different (P = 5 %; Tukey's test). b – acclimatized plantlet in field, 8 months after transfer

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