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Micropropagation of *Barleria prionitis* L. var. *dicantha*: an ethnomedicinal plant

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Abstract
Barleria prionitis var. *dicantha* Blatt & Hallb is an important endemic, ethno medicinal plant of Rajasthan, commonly known as Vajaradanti. It is especially well known for its antidontalgic properties. It is a rich source of glycosides, steroids, tannins and flavonoids. Natural habitat loss due to urbanization, high habitat specificity and exploitation for medicinal values places considerable pressure on native population of this endemic endangered plant. There is a need for development of non-conventional methods for propagation and conservation of *B. prionitis* var. *dicantha*. The present study was undertaken with the aim to set up a protocol for *in vitro* propagation of this medicinally important plant. For this, nodal segments were obtained from *in vitro* raised seedling on half strength MS medium supplemented with 2.88 µM GA_3. For axillary bud break, nodal segments were inoculated on different concentrations of BA, Kn or TDZ supplemented MS medium. Maximum (75.5%) bud break was obtained on MS medium supplemented with 8.88 µM BA + additives with an average of 3.82±0.13 shoots explant^{-1} and 1.74±0.08 cm shoot length. The elongated shoots were excised from mother plant and further multiplied on MS medium supplemented with 4.44 µM BA. For rooting *in vitro* raised shoots were cultured on MS medium supplemented with different concentrations IBA or IAA. The maximum rooting was 75.5% obtained on 2.46 µM IBA with 4±0.13 roots explant^{-1} and 3.44±0.04 cm root length. The *in vitro* raised plantlets were successfully hardened and acclimatized in polybags at shade house.
Key words: Antidontalgic; Axillary bud break; Vajaradanti; Shoot multiplication; *In vitro* rooting.

Abbreviations
BA: 6-Benzylaminopurine; GA_3: Gibberellic acid; IAA: Indole acetic acid; IBA: Indole-3-butyric acid; Kn: Kinetin; MS: Murashige and Skoog medium; PGRs: Plant growth regulators; RH: Relative humidity; TDZ: Thidiazuron, FYM: Farm yard manure.

Introduction

Barleria prionitis var. *dicantha* (Vajaradanti) belongs to the family Acanthaceae, is a multipurpose medicinal plant. It is well known for treating bleeding gums and toothache because of which it is known as Vajaradanti, which means saw-edged in sanskrit. The whole plant parts (leaves, flowers and roots) are used in traditional indian medicines for treatment of catarrhal affections, urinary infection, jaundice, glandular swellings, migraine, oedema, haemoptysis, reduce obesity (Khare 2004; 2007). Whole-plant extracts of *Barleria* contain iridoid glycosides, barlerin and verbascoside which have potent activity against respiratory syncytial virus and in herbal medicine (Chen et al. 1998). Anti–spermatogenic (Verma et al. 2005), anti-dibertic (Dheer and Bhatnagar 2010), antidepresent (Gangophadhyay et al. 2012), immunostimulatory (Ghule and Yeole 2012), hepatoprotective (Singh et al. 2005) properties were reported. The phytochemicals found in *B. prionitis* have inhibitory effect against GTS (glutathione S-transferase) which is considered responsible for decreasing the effectiveness of anticancer or antiparasitic agents also have inhibitory effect against acetylcholinesterase. They also have potential application in the treatment of cardiac disorder and Alzheimer's disease (Ata et al. 2007). Maji et al. (2011) found extract of whole plant shows inhibition of hyposaline induced erythrocyte membrane hemolysis and induced mast cell degranulation.

Increasing human and livestock populations have affected the status of wild plants particularly those used in herbal medicines (Singh et al. 2009). Due to overexploitation for medicinal and cosmetic uses along with habitat destruction and endemic nature of *B. prionitis* var. *dicantha* is now categorized as endangered (Khan et al. 2003; Pandey et al. 2012; Lone et al. 2013). Conventionally *B. prionitis* is propagated mainly through the seeds and shoot cutting. However, germination of seeds is poor (Menges and Gordon 1996; Siyol and Sharma 2009) and shoot cutting solely relies on season for multiplication, which makes it an in-efficient way for the conservation of this medicinally important plant. Moreover, these conventional methods of propagation means cannot fulfill the demand for medicinal and cosmetic uses. Therefore, there is need for the establishment of an efficient micropropagation method for the conservation of *B. prionitis* var. *dicantha*.

Plant tissue culture is a useful tool for the conservation and large-scale propagation of medicinally important and endangered plants (Singh et al. 2012; Thyagarajan and Venkatachalam 2012). In tissue culture, plant growth regulators are important media components in determining the development and developmental pathway of the plant cells. Growth regulators are used in different proportions to break dormancy and enhance shoot formation since it is well demonstrated that the apical dormancy is under control of these growth regulators (Madhulatha et al. 2004). Cytokinins such as benzylaminopurine (BA) and kinetin are known to reduce the apical meristem dominance and induce both auxiliary formation from meristematic explants in many medicinal plants *Glycyrrhiza glabra* (Arya et al. 2009b) *Azadirachta indica* (Gehlot et al. 2014), *Terminalia arjuna* (Choudhary et al. 2015), *Stevia rebaudiana* (Rathi et al. 2015). Auxins such as indol-3-butyric acid (IBA) have been reported to promote plant rooting *in vitro*, *Dalbergia sissoo* (Arya et al. 2013), *Eucalyptus* hybrids FRI-5 and FRI-14 (Arya et al. 2009a), *Bacopa monnieri* (Sharma et al. 2010).

There has been progress in tissue culture studies in many Acanthaceae members such as *Adhatoda vasica* (Abhyankar and Reddey 2007), *Dipteracanthus prostates* (Robert et al. 2012), *Adhatoda beddomei* (Panigrahi 2014), *Clinacanthus nutans* (Chen et al. 2015). Preliminary research on *in vitro* callus production in *B. prionitis* L. has been reported by some authors (Premjet et al. 2010; Shukla et al. 2011).

There is no previous report about micropropagation of *B. prionitis* var. *dicantha*. However, reports are there on micropropagation of *B. prionitis* (Lone et al. 2011; 2013). The main objective of present study was to develop efficient protocol for micropropagation of *B. prionitis* var. *dicantha* using axillary nodal segments of juvenile plants.

Material and methods

Plant material and surface sterilization

Seeds of *B. prionitis* var. *dicantha* were collected from plants in Rao Jodha Park, Jodhpur (Fig. 1A). Healthy seeds were selected and soaked for 2 days in distilled water. After two days, these seeds were surface sterilized by passage through a mild detergent, Tween 80 for 5 min, followed by washing with distilled water. Before transferring the seeds to laminar flow, these seeds were pretreated with Streptomycin (0.1%) and fungicide Carbendazim (0.1%) for 5 min and were then rinsed 3-4 times with autoclaved distilled water. On laminar flow, these seeds were surface sterilized with 0.1% $HgCl_2$ for 3 min and rinsed 3-4 times with autoclaved distilled water. Seed coat of surface sterilized seeds was removed, inoculated on half strength MS medium supplemented with 2.88 µM GA_3 and incubated in growth room. Nodal segments (1.5-2 cm in length) were obtained from 4-week-old aseptic seedlings were used as explants.

Figure 1. General aspect of micropropagation protocol of *B. prionitis* var. *dicantha*. (A) Mature plant of *B. prionitis* var. *dicantha*. Bar = 10 cm. (B) Axillary bud break on MS + 8.88 µM BA. Bar = 1 cm. (C) *In vitro* shoot multiplication on MS + 4.44 µM BA. Bar= 0.5 cm. (D-E) *In vitro* rooting on MS + 2.46 µM IBA. Bar = 1 cm. (F) *In vitro* hardening. Bar = 1 cm. (G) Hardening in mist chamber. Bar = 2 cm. (H) Tissue culture raised plants of *B. prionitis* var. *dicantha*. Bar = 2 cm.

Nutrient medium and culture condition

MS (Murashige and Skoog 1962) medium supplemented with different concentrations of BA (0, 2.22, 4.44, 8.88, 13.32 or 17.76 µM), Kn (0, 2.32, 4.65, 9.28, 13.92 or 23.20 µM) or TDZ (0, 0.04, 0.22, 0.45, 2.27 or 4.54 µM) were used for axillary bud break, MS medium, without cytokinin, served as control. All culture media were supplemented with 3 % (w/v) sucrose and additives (100 mg L^{-1} ascorbic acid, 50 mg L^{-1} citric acid and 50 mg L^{-1} adenine sulphate). Agar-agar (Hi-Media™, Mumbai, India) was added to media as a gelling agent at the concentration of 0.8% (w/v). The pH of the medium was adjusted to 5.8±0.2 with 1N NaOH or HCl, the medium was autoclaved at and 121°C for 15 min. All the cultures vessels (test tube 25×150 mm and 20-25 mL, conical flask 100 and 150 mL), 1,600 lux intensity light via white cool florescent tubes (Philips™, India), 16h/8h light/dark period respectively were maintained under aseptic condition.

In vitro shoot multiplication

After each harvest of elongated shoot for further multiplication the explants were subcultured on the same medium. For multiplication, the *in vitro* raised shoots were excised from mother explants. These shoots were subcultured on different cytokinin, namely BA (0, 2.22, 4.44, 8.88, 13.32 and 17.76 µM), Kn (0, 2.32, 4.65, 9.28, 13.92 or 23.20 µM) or TDZ (0, 0.04, 0.22, 0.45, 2.27 or 4.54 µM). Media constituent and culture conditions were similar to shoot induction media.

In vitro rooting

Well developed *in vitro* raised shoots of 1-2 cm length were used for various *in vitro* rooting experiments. Individual shoots were carefully separated and transferred to MS medium enriched with IAA (0.00-9.84 µM) or IBA (0.00-11.41 µM) along with additives ascorbic acid (100 mg L^{-1}), citric acid (50 mg L^{-1}). Effect of medium strength (half strength or full strength) was also studied for *in vitro* rooting. The rooted plantlets were removed from culture vessel and their roots were thoroughly washed with autoclaved distilled water to remove all remains of medium. These were transferred to jam bottles (400 mL) containing autoclaved soilrite moistened with 10-15 mL half strength MS salts. The bottles were capped and kept for 2-3 week under growth room conditions for *in vitro* hardening.

Hardening and acclimatization of in vitro raised plantlets

After 2-3 weeks of *in vitro* hardening, jam bottles containing rooted plantlets were transferred to mist chamber near the pad section (high RH 80-90% and low temperature 28±2ºC). One week after the transfer of *in vitro* raised plantlets to the mist chamber the caps of jam bottles were increasingly loosened to reduce RH. These plantlets were shifted from the pad section towards the fan section to provide growing conditions of low humidity and higher temperature. Plants were transferred to polybags containing a mixture soil + sand + FYM (1:1:1, v/v). Farm Yard Manure (FYM) is a decomposed mixture of cattle dung and urine with residues from the fodder. These hardened plants were then shifted to shade house for acclimatization.

Experimental design and data analysis

After 4 week in culture, axillary bud break, shoot multiplication shoot number, shoot length and in rooting culture root number and root length were recorded. Data were analyzed through General linear model (GLM) through one way analysis using statistical package for social science (SPSS 17.0). BA, Kn, TDZ, IAA and IBA were taken as an independent (fixed) factor whereas axillary bud break and proliferation, shoot number and shoot length (cm), root length, root number and rooting percentage (%) were taken as dependent factor. Duncan multiple range test (DMRT) at $P \leq 0.05$ was used to compare the means. All the experiments were conducted with 15 replicate per treatment. Each experiment was repeated three times.

Results and discussion

Axillary bud break and in vitro shoot proliferation

One of the main functions of cytokinin is to release axillary buds from suppression due to apical dominance thus initiating axillary bud induction and proliferation. The morphogenic response of explants to various cytokinins (BA, Kn, TDZ) was evaluated. Nodal explants cultured on growth regulator-free medium provide low percentage of bud break. Fatima and Anis (2012) reported that the presence of growth regulators in the medium promotes bud induction and development. Addition of cytokinin is essential to induce bud break and multiple shoot formation from the explants (Lodha et al. 2015). Among three cytokinins tested maximum bud break response was obtained on BA.

The requirement for exogenous cytokinin and auxin in bud differentiation varies among tissue and apparently depends on the endogenous level of the two hormones. Number of explants response for bud break increases along with BA concentrations, up to optimal level. BA at 8.88 µM showed the highest axillary bud break (75.5%) with 3.82±0.13 shoots explant^{-1} and 1.74±0.08 cm shoot length (Table 1; Fig. 1B). The frequency of axillary bud break and *in vitro* shoot proliferation was relatively low when the medium is supplemented with Kn or TDZ (Table 2 and 3). The efficiency of BA for shoot induction was documented for medicinal plants of acanthaceae family *Adhatoda vasica* (Abhyankar and Reddy 2007), *Beloperone plumbaginifolia* (Muthuramalingam et al. 2014), *Andrographis paniculata* (Dandin and Murthy 2012), *Graptophyllum pictum* (Koilpillai and Wilson 2010).

Lone et al. (2013) reported that use of BA along with TDZ is optimal for the shoot induction of *B. prionitis*, which is different from the present finding.

In vitro shoot multiplication

Shoot multiplication is the major criteria for successful commercial micropropagation. The multiplication rate achieved at this stage determines the feasibility of *in vitro* propagation of a given plant species. High concentration of cytokinin from the bud induction medium may accumulate in tissues, which may suppress growth and multiplication of subcultures (Malik et al. 2005). Therefore the *in vitro* raised shoots were transferred to lower concentration of cytokinins (BA, Kn and TDZ).

In the present study, BA proved its superiority over other cytokinins, it showed effect on number of shoot produced and shoot length. The higher multiplication 3.73±0.13 shoots explant^{-1} and shoot length 2.85±0.03 cm was observed on MS medium supplemented with 4.44 µM BA (Table 4, 5 and 6; Fig. 1C). Significant role of BA in shoot multiplication was also reported in *Andrographic echioides* (Hemalatha and Vadivel 2010), *Adhatoda beddomei* (Panigrahi 2014), *Crossandra infundibuliformis* (Girija et al. 1999).

Table 1. Effect of different BA concentration on *in vitro* axillary bud break of *B. prionitis* var. *dicantha* after four weeks of culture.

BA (µM)	Bud break (%)	Shoots (explant^{-1})	Shoot length (cm)
Control	15.5^{ds}	1.14±0.14^{d}	0.36±0.04^{d}
2.22	35.5^{c}	1.56±0.12^{d}	0.95±0.03^{c}
4.44	53.3^{bc}	2.62±0.14^{c}	1.26±0.04^{b}
8.88	75.5^{a}	3.82±0.13^{a}	1.74±0.08^{a}
13.32	62.2^{ab}	3.39±0.09^{b}	1.56±0.06^{a}
17.76	48.8^{bc}	2.40±0.10^{c}	0.88±0.06^{c}
P value	≤ 0.001	≤ 0.001	≤ 0.001

Mean ± standard error in the same column followed by different letters is different at $P \leq 0.05$ (DMRT).

Table 2. Effect of different Kn concentration on *in vitro* axillary bud break of *B. prionitis* var. *dicantha* after four weeks of culture.

Kn (µM)	Bud break (%)	Shoots (explant^{-1})	Shoot length (cm)
Control	15.5^{e}	1.00±0.00^{d}	0.38±0.03^{e}
2.32	24.4^{de}	1.27±0.14^{cd}	0.84±0.04^{d}
4.69	35.5^{cde}	1.68±0.15^{c}	1.06±0.04^{c}
9.24	46.6^{ab}	2.14±0.14^{b}	1.30±0.03^{b}
13.93	62.2^{a}	2.67±0.12^{a}	1.53±0.03^{a}
18.58	37.7^{cd}	2.35±0.11^{ab}	1.12±0.04^{c}
P value	≤ 0.001	≤ 0.001	≤ 0.001

Mean ± standard error in the same column followed by different letters is different at $P \leq 0.05$ (DMRT).

Table 3. Effect of different TDZ concentration on *in vitro* axillary bud break of *B. prionitis* var. *dicantha* after four weeks of culture.

TDZ (µM)	Bud break (%)	Shoots (explant^{-1})	Shoot length (cm)
Control	13.3^{c}	1.00±0.00^{d}	0.50±0.05^{d}
0.04	28.8^{bc}	1.23±0.12^{cd}	0.84±0.04^{c}
0.22	40.0^{ab}	1.50±0.12^{bc}	1.15±0.06^{b}
0.45	57.7^{a}	2.26±0.11^{a}	1.50±0.05^{a}
2.27	48.8^{ab}	1.81±0.14^{b}	1.28±0.05^{b}
4.54	37.7^{ab}	1.35±0.11^{cd}	0.93±0.03^{c}
P value	≤ 0.001	≤ 0.001	≤ 0.001

Mean ± standard error in the same column followed by different letters is different at $P \leq 0.05$ (DMRT).

Table 4. Effect of different BA concentration on *in vitro* shoot multiplication of *B. prionitis* var. *dicantha* after four weeks of culture.

BA (µM)	Shoots (explant^{-1})	Shoot length (cm)
Control	1.22±0.06^{f}	1.26±0.02^{f}
2.22	2.02±0.09^{e}	1.49±0.04^{e}
4.44	3.73±0.10^{a}	2.85±0.03^{a}
8.88	3.42±0.08^{b}	2.63±0.03^{b}
13.32	3.15±0.06^{c}	2.39±0.02^{c}
17.76	2.66±0.10^{d}	1.93±0.02^{d}
P value	≤ 0.001	≤ 0.001

Mean ± standard error in the same column followed by different letters is different at $P \leq 0.05$ (DMRT).

Table 5. Effect of different Kn concentration on *in vitro* shoot multiplication of *B. prionitis* var. *dicantha* after four weeks of culture.

Kn (µM)	Shoots (explant^{-1})	Shoot length (cm)
Control	1.24±0.06^{e}	1.33±0.03^{c}
2.32	1.57±0.09^{d}	1.35±0.02^{c}
4.69	2.11±0.09^{bc}	1.42±0.02^{c}
9.24	2.82±0.10^{a}	2.26±0.04^{a}
13.93	2.28±0.06^{b}	1.54±0.03^{b}
18.58	1.88±0.09^{c}	0.97±0.03^{d}
P value	≤ 0.001	≤ 0.001

Mean ± standard error in the same column followed by different letters is different at $P \leq 0.05$ (DMRT).

In vitro rooting

The ability of plant tissue to form roots depends on interaction of many endogenous and exogenous factors. The role of auxin in root development was established and reviewed by Torrey (1965; 1976) and Aloni (2004). Since there is enough residual cytokinin present in shoot therefore, little or no cytokinin is required in rooting medium. In present study, low rooting percent was observed in absence of auxins and maximum rooting response was observed when IBA was incorporated. Different IBA concentration significantly affects number of roots produced and their length. Half strength of MS medium supplemented with 2.46 µM IBA gives maximum rooting response with mean root number 4±0.13 and 3.6±0.04 cm root length (Table 7 and 8; Fig. 1D and E). Compare to IAA, IBA was found to be a better auxin for *in vitro* root induction. Similar result has

been reported earlier in other medicinal plants *Adhatoda vasica* (Mandal and Laxminarayan 2014), *Indoneesiella ecohides* (Wilson et al. 2010), *Dipteracanthus prostrates* (Robert et al. 2012), *Hygrophila polysperma* (Karatas et al. 2013), *Andrographis paniculeta* (Al-Mamun et al. 2015), *Asteracanthas longifolia* (Kumar and Nandi 2015), *Clinacanthus nutans* (Chen et al. 2015).

Table 6. Effect of different TDZ concentration on *in vitro* shoot multiplication of *B. prionitis* var. *dicantha* after four weeks of culture.

TDZ (µM)	Shoots (explant^{-1})	Shoot length (cm)
Control	1.37±0.07^{d}	1.36±0.02^{e}
0.04	2.15±0.10^{c}	1.49±0.03^{d}
0.22	2.46±0.07^{b}	1.71±0.02^{c}
0.45	2.97±0.11^{a}	2.32±0.01^{a}
2.27	2.53±0.08^{b}	1.95±0.02^{b}
4.54	2.28±0.10^{bc}	1.65±0.03^{c}
P value	≤ 0.001	≤ 0.001

Mean ± standard error in the same column followed by different letters is different at $P \leq 0.05$ (DMRT).

Table 7. Effect of different concentration IBA of on *in vitro* rooting of *B.prionitis* var. *dicantha* after four weeks of culture.

IBA (µM)	Rooting (%)	Roots (explant^{-1})	Root length (cm)
Control	17.7^{d}	1.25±0.16^{e}	0.90±0.11^{f}
0.49	48.8^{bc}	2.72±0.14^{cd}	2.42±0.01^{c}
2.46	75.5^{a}	4.00±0.13^{a}	3.44±0.04^{a}
4.92	66.6^{ab}	3.46±0.10^{b}	2.79±0.03^{b}
7.38	60.0^{ab}	3.11±0.13^{bc}	2.19±0.05^{d}
9.84	33.3^{c}	2.40±0.16^{d}	1.63±0.07^{e}
P value	≤ 0.001	≤ 0.001	≤ 0.001

Mean ± standard error in the same column followed by different letters is different at $P \leq 0.05$ (DMRT).

Table 8. Effect of different IAA concentration on *in vitro* rooting of *B.prionitis* var. *dicantha* after four weeks of culture.

IAA (µM)	Rooting (%)	Roots (explant^{-1})	Root length (cm)
0.00	17.7^{d}	1.25±0.16^{e}	0.88±0.09^{e}
0.57	66.6^{ab}	2.43±0.09^{c}	2.45±0.03^{b}
2.85	71.1^{a}	3.74±0.13^{a}	2.94±0.04^{a}
5.70	55.5^{ab}	2.88±0.13^{b}	2.55±0.06^{b}
8.56	42.2^{bc}	2.52±0.11^{bc}	2.21±0.06^{c}
11.41	30.0^{c}	1.76±0.16^{d}	1.66±0.06^{d}
P value	≤ 0.001	≤ 0.001	≤ 0.001

Mean ± standard error in the same column followed by different letters is different at $P \leq 0.05$ (DMRT).

Hardening and acclimatization

The tissue culture raised plantlets are heterotrophic in their mode of nutrition and cannot withstand harsh environmental without proper hardening and acclimatization. Hardening in low carbohydrate medium and exposure to high level of light intensity is recommended to force the *in vitro* regenerated plants to rely on their photosynthetic apparatus for nutrition. Such hardened plantlets when transferred to field conditions gave better results as compared to non hardened (Lavanya et al. 2009).

In order to expose plantlets to outer environmental conditions, culture vessels were moved from high humidity and low temperature to low humidity and high temperature zone. This allows plants to acclimatized and withstand in harsh outer environmental condition (Fig. 1F). These plants were transferred to polybags containing FYM: sand: soil in ratio 1:1:1 mixture, were acclimatized successfully under the shade with 72% survival rates recorded after 60 days (Figs. 1G and H). Gradual acclimatization and hardening enhanced the capacity of plantlets to withstand water loss and allow them to carry out photosynthesis which ultimately increase survival rate of plantlets in the environmental conditions.

Conclusion

In this research article, for the first time a protocol for micropropagation of *B. prionitis* var. *dicantha* from seedling raised nodal explants has been developed. The micropropagation of *B. prionitis* var. *dicantha* was remarkably affected by growth regulators and their concentration. The developed protocol could support conservation of plant species from indiscriminate exploitation from its natural resources, ultimately enabling to keep pace with commercial needs.

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