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Research Article

IN VITRO CLONAL PROPAGATION OF *DIPTERACANTHUS PROSTRATUS* (POIR.) NEES. – A VALUABLE MEDICINAL PLANT

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ABSTRACT

High frequency *in vitro* plantlet regeneration protocol for *Dipteracanthus prostratus* has been developed in this study. Nodal shoot segments were used as explants to establish the cultures. The explants were sterilized using 0.1% $HgCl_2$ solution for 4-5 min. The Murashige and Skoog (MS) medium augmented with 1.0 mg l^{-1} 6-benzylaminopurine (BAP) was reported suitable for bud break from the nodal meristems of the explants. About 4.6 shoots with 3.7 cm length were induced per explant on this medium combination. The shoots were multiplied (31.7 shoots with 5.6 cm length) on MS medium supplemented with 0.5 mg l^{-1} each of BAP and Kinetin (Kin). The healthy and sturdy shoots were rooted *in vitro* on half-strength MS medium fortified with 1.5 mg l^{-1} indole-3-butyric acid (IBA). The rooted plantlets were hardened in the greenhouse using soilrite first in the bottles for 4 week and then in the paper cups for 2 weeks. The hardened plants were transferred to the earthen pots and finally shifted to the natural fields. About 96% plantlets were survived in the field conditions.

The developed protocol could be used for mass propagation of this important medicinal plant. The superior planting material can be supplied to the farmers and drug manufactures.

Key words: *Dipteracanthus prostratus*, micropropagation, rooting, hardening.

INTRODUCTION

Dipteracanthus prostratus (Poir.) Nees. (Synonym *Rullia prostrata*) is an indigenous medicinal plant of India belongs to the highly developed natural group, family Acanthaceae. It is a prostrate perennial herb with profuse branches, up to 50 cm tall, woody at base, locally known as Haadjud by the tribal community. The stem is greenish and angular, leaves are 5-10 mm long, lamella elliptic ovate and densely pubescent on both the surfaces. Flowers are sessile, 2-4 cm long, pale white in color, 2-3 in cymes, and capsule fruits with 8-10 seeds. The plant usually prefers to grow in moist and shady places. It is widely distributed in Arica, Srilanka, Pakistan and throughout India [1].

The plant is commonly known as bell weed and the leaves are used as vegetable [2]. This plant gains significance due to its medicinally important properties like anticancer, hypoglycemic, anti-inflammatory, diuretic, antileishmanial and antimicrobial activities [3-5]. Historically this plant is used to cure gonorrhea, hypoglycemia, eye disease and ear diseases [6-8]. The important bioactive compounds isolated from this plant are glycosides, phenolic compounds, saponins, tannins, flavonoids, gums, alkaloids and mucilage. The roots of bell weed are edible and used in preparation of local drinks [9]. The whole plant juice is used as antidote for snakebite by the tribal of the western ghats of south India [10].

These varied uses have increased utilization and exploitation of this plant for medicinal purposes. As a result, natural stands of *D. prostratus* are fast disappearing due to indiscriminate collection. The plants grow wild in forests and there is no propagation system available to replenish the natural population. Micropropagation techniques are of special use for the propagation and conservation of this valuable medicinal plant. The *in vitro* work on *D. prostratus* species is largely deficit in literature at national and international level [11]. The present study aims to derive stable reproducible micropropagation protocol using nodal shoot segments of *D. prostratus.*

MATERIALS AND METHODS

Plant material, explants selection and surface sterilization

Healthy plants of *D. prostratus* were collected from the Coromandel Coast of the South India and maintained in the greenhouse. Nodal explants with 2-3 nodes were selected from the greenhouse grown plants during the months of June to December. The explants were first treated with 0.1% (w/v) Bavistin (a systemic fungicide; BASF India Limited, Mumbai, India) for 6-8 min and then surface sterilized with 0.1% (w/v) $HgCl_2$ for 4-5 min, which is further washed with sterile distilled water for 5-7 times under aseptic conditions in a laminar air flow chamber.

Establishment of culture and characterization of culture environment

The sterilized nodal explants were inoculated on Murashige and Skoog medium [12] containing 8.0 g agar (Bacteriological grade, Himedia, India), 30 g sucrose and BAP or Kin with different ranges (0.5–4.0 mg l^{-1}) for shoot bud induction. Further MS medium was enriched by the incorporation of additives namely ascorbic acid (50 mg l^{-1}) and 25 mg l^{-1} each of citric acid, adenine sulphate and L-arginine. The pH of the medium was attuned to 5.8 ± 0.02 using 1N NaOH or HCl before autoclaving at 1.1 kg cm^{-2} pressure and 121ºC for 15 min. Nodal explants were inoculated vertically in culture tubes for *in vitro* shoot bud induction. Cultures were initially incubated (for 2-3days) under diffused light, where 20-25 µmol m^{-2} s^{-1} spectral flux photon density (SFPD) light intensity was maintained for bud break. Thereafter, these were transferred to photon rich area of culture room and maintained at12/12 hr light/dark photo regims per day with the light intensity of 40-50 µmol m^{-2} s^{-1} at 28 ± 2ºC temperature and 60% relative humidity. The light intensity was provided using cool and white fluorescent tubes (Philips, India).

Proliferation of shoots *in vitro*

The *in vitro* regenerated shoot buds were multiplied by repeated transfer of the mother explants with and without *in vitro* raised shoots on to fresh MS medium. The *in vitro* regenerated shoots were excised with 3-5 nodes and subcultured on fresh nutrient medium for further multiplication of shoots. The MS medium fortified with additives and various concentrations and combinations of cytokinins (BAP, 0.5-2.0 mg l^{-1} and/or Kn, 0.1-1.0 mg l^{-1}) and auxin (IAA 0.1-1.0 mg l^{-1}) were formulated for shoot multiplication. Cultures were repetitively subcultured after an interval of 4 weeks.

In vitro rhizogenesis

Healthy shoots with 5-6 nodes of multiplication stage were excised and transferred to different strength (Full, half and one-fourth strength) of

semisolid MS medium augmented with different auxins with varying concentration (IAA and IBA, 0.5-3.0 mg l^{-1}) and 100 mg l^{-1} activated charcoal for *in vitro* root induction. The rooting medium was further enriched with additives. After 4-5 week of root development the *in vitro* rooted shoots were separated from culture vessels and adhered medium was removed with distilled water. These plantlets were transplanted to steam sterilized soilrite® (a mix up of perlite, peat moss and exfoliated vermiculite, Kel Perlite, Bangalore, India) in polycarbonate capped glass bottles (70 × 135 mm) moistened with one-fourth strength of nutrient MS basal salt solution.

Acclimatization, hardening and transplantation

In vitro rooted plantlets were placed in the greenhouse to maintain high humidity and low temperature required for acclimatization. The caps of bottles were steadily loosened after 2 week and completely removed after 5-6 week to expose the plantlets to the greenhouse conditions. The bottles were progressively shifted to low humidity and high temperature area of the greenhouse for gradual hardening. The hardened plantlets were transplanted to nursery poly-bags containing garden soil: farmyard manure: soilrite® in 3:1:1 ratio and finally transferred to the field after another 4 week.

Experimental design, data collection and statistical analysis

All the experiments were carried out in a completely randomized block design for single factor experiments. Each treatment has 10 replicates and all the experiments were repeated thrice. The data were recorded after 4 week of culture and subjected to statistical analysis by ANOVA using SPSS ver. 16 (SPSS Inc., Chicago, USA). The significance of differences among mean values was calculated by Duncan's multiple range tests at $P<0.05$.

RESULTS AND DISCUSSION

Induction of shoots from the nodal meristems

The bud break and shoot initiation was achieved from the nodal meristems within 7 days after inoculation. Explants procured from young branches in the month of November responded better revealed the significance of season of collection of explants. Seasonal response of explants played important role in culture induction in number of plant species [13-15]. Primary incubation of cultures in diffused light after inoculation was supported new shoot bud initiation and further growth. Bud breaking response in diffused light incubation has also been reported in *Rauvolfia serpentina* [16] and *Caralluma edulis* [17]. Significant differences in regeneration frequency (%) were observed among the explants on different concentration of BAP and Kin. Comparatively highest number of shoot buds was induced on MS medium supplemented with 1.0 mg l^{-1} BAP (Fig. 1A and Table 1). Maximum 4.6 shoots with 3.7 cm length were induced from each node within 4 week of incubation with this concentration.

The importance and superiority of BAP over other hormones in bud breaking was reported and presented by Lodha et al. [15] in *Cadaba fruticosa* and Shekhawat et al. [18] in *Morinda coreia*. The higher concentration of BAP in the medium has proved inhibitory in bud breaking. MS medium devoid of growth hormones and low concentration of Kin did not result in shoot bud regeneration. These findings are contrast from the observations of the Robert et al. [11] where they obtained maximum 80% shoot response on 1.0 mg l^{-1} Kin.

Table 1:Effect of cytokinins (BAP and Kin) concentration on bud breaking response from nodal meristem of *D. prostratus.*

Conc. of BAP (mg l^{-1})	Conc. of Kin (mg l^{-1})	Response (%)	Shoot number (Mean±SD)	Shoot length (cm) (Mean±SD)
0.0	0.0	0.00	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
0.5	-	27.4	2.0 ± 0.4^{c}	3.0 ± 0.2^{d}
1.0	-	89.0	4.6 ± 0.7^{e}	3.7 ± 0.5^{e}
1.5	-	44.6	3.1 ± 1.1^{d}	2.5 ± 0.0^{c}
2.0	-	40.9	2.0 ± 1.0^{c}	2.8 ± 0.9^{c}
3.0	-	39.2	1.3 ± 0.3^{b}	2.2 ± 0.1^{c}
-	0.5	12.0	0.0 ± 0.0^{a}	1.5 ± 0.4^{b}
-	1.0	46.8	1.6 ± 0.5^{b}	2.2 ± 0.0^{c}
-	1.5	43.1	2.0 ± 1.2^{c}	1.4 ± 0.5^{b}
-	2.0	36.4	1.3 ± 0.6^{b}	1.0 ± 0.0^{b}
-	3.0	32.6	1.0 ± 0.4^{b}	1.0 ± 0.7^{b}

Note: Mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and significance variation between the concentrations was studied using DMRT at 0.05% level.

Multiplication of shoots by repetitive transfer

Shoot multiplication was enhanced by subsequent transfer of *in vitro* regenerated shoots on to MS medium with various combinations of cytokinins. Repeated subculture of mother explants on fresh medium responded positively up to 4th subculture and thereafter gradually decreased in number of shoots due to apical dominance of nodal shoots. Subculture of *in vitro* regenerated shoots with 3-4 nodes responded better than the earlier experiment (Fig. 1B and 1C). MS medium augmented with 0.5 mg l^{-1} each of BAP and Kin resulted best among the different concentrations experienced. Maximum number of healthy shoots (31.7 shoots with 5.6 cm) was obtained with this concentration when additives incorporated in the medium (Table 2). Additives ascorbic acid and citric acid play antioxidant role and adenine sulphate and L-arginin are nitrogen reducing agents which assist in cell division along with the cytokinins in the medium [15]. Combined effect of BAP and Kin for better multiplication of shoots was studied in *Passiflora foetida* [19, 20]. Robert et al. [11] were able to multiplied 2.66 shoots only with 3.96 cm length on MS medium supplemented with 1.0 mg l^{-1} Kin and 0.2 mg l^{-1} NAA.

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Fig. 1A: Induction of shoots from the nodal meristems on MS medium. Fig. 1B and 1C: Multiplication and elongation of shoots *in vitro*. Fig. 1D: *In vitro* rooting in *D. prostratus*. Fig. 1E: Hardening of the plantlets with soilrite in bottles and then paper cups. Fig. 1G and 1H: Hardened plants in pot and transplanted to the field.

Table 2: Effect of different concentrations and combinations of cytokinins (BAP+ Kn) on shoot multiplication of *D. prostratus*.

Conc. of BAP (mg l^{-1})	Conc. of Kin (mg l^{-1})	Shoot numbers (Mean ± SD)	Shoot length (cm) (Mean ± SD)
0.1	0.1	20.1 ± 1.6^{c}	4.0 ± 0.3^{c}
0.5	0.5	31.7 ± 2.3^{g}	5.6 ± 0.7^{e}
1.0	0.5	26.4 ± 0.6^{f}	4.9 ± 0.1^{d}
1.5	0.5	25.0 ± 0.3^{e}	4.1 ± 0.4^{c}
2.0	0.5	22.7 ± 1.2^{d}	3.7 ± 0.8^{b}
2.5	0.5	17.5 ± 0.9^{b}	3.0 ± 0.3^{a}
3.0	0.5	10.2 ± 1.0^{a}	3.2 ± 0.6^{a}

Note: Mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and significance variation between the concentrations was studied using DMRT at 0.05% level.

***In vitro* root induction from the cut ends of the shoots**

Among the rooting medium formulated, half strength MS basal salts medium with 15 g l^{-1} sucrose fortified with 1.5 mg l^{-1} IBA and 100 mg l^{-1} activated charcoal produced maximum number of roots (5.9 roots with 4.3 cm length) within 4-5 week of incubation (Fig. 1D and Table 3). IBA was observed prominent over IAA in terms of response (%) and induction of roots in this study. The positive effect of IBA in rooting has been reported in many plant species like *Eulophia nuda* [21], *Caralluma edulis* [17] and *Morinda coreia* [18]. The reduced strength of MS salts (half strength) enhanced rooting frequency by creating stress in the nutrient environment, this was also confirmed in *Psidium guajava* [22] and *M. coreia* [18]. Robert et al. [11] obtained 3.63 roots per shoot on full strength MS medium augmented with 0.5 mg l^{-1} IBA.

Table: 3: Effect of IBA and IAA concentrations and ½ strength MS medium on rhizogenesis *in vitro*.

Conc. of IBA (mgl^{-1})	Conc. of IAA (mgl^{-1})	Response (%)	Number of root (Mean ± SD)	Length of root (cm) (Mean ± SD)
0.0	-	00.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
0.5	-	56.1	5.0 ± 0.2^{e}	2.6 ± 0.6^{c}
1.0	-	79.4	5.9 ± 0.7^{g}	4.3 ± 0.3^{f}
1.5	-	90.7	5.3 ± 0.9^{f}	5.8 ± 0.8^{i}
2.0	-	81.3	4.6 ± 0.2^{d}	5.0 ± 0.5^{h}
3.0	-	72.0	4.2 ± 0.5^{c}	4.7 ± 0.2^{g}
-	0.5	33.8	3.5 ± 0.3^{b}	1.9 ± 0.9^{b}
-	1.0	47.2	4.1 ± 0.0^{c}	3.0 ± 0.4^{d}
-	1.5	69.5	5.2 ± 0.2^{f}	3.6 ± 0.6^{e}
-	2.0	60.8	4.7 ± 0.5^{e}	4.0 ± 0.1^{e}
-	3.0	50.4	4.0 ± 0.8^{c}	2.3 ± 0.4^{b}

Note: Mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and significance variation between the concentrations was studied using DMRT at 0.05% level.

Acclimatization, hardening and field transfer of plantlets

The *in vitro* rooted shoots were acclimatized successfully under the greenhouse conditions. The rooted plantlets were placed in the glass bottles containing sterilized soilrite® (Fig. 1E) and maintained in the greenhouse to provide evaporative cooling environment of high humidity and low temperature at initial stage. The caps of the bottles were gradually loosened after 2 week and completely removed after 6 week to adopt the plantlets to the greenhouse conditions. In next 3-4 week the plantlets were transplanted to the paper cups containing soilrite® and shifted to the low humidity and high temperature zone of the greenhouse in order to expose plantlets to *ex vitro* conditions (Fig. 1F). Proper hardening before transplanting *in vitro* regenerated plants has emerged as a serious concern, since it is essential in deriving successful micropropagation protocol for any plant species [23, 24]. The greenhouse hardened plantlets were further transferred to the earthen pots (Fig. 1G) containing garden soil, farmyard manure and soilrite® (3:1:1) and finally transferred to the field (Fig. 1H). About 92% plants were successfully survived without any adverse effect of field conditions.

CONCLUSION

The developed micropropagation protocol is highly reproducible which can be used to restore the depleting population of this important medicinal plant in the forest. The rate of shoot multiplication is very high in present study; simultaneously the rooting has been achieved with good response. The plantlets were hardened and successfully field transferred.

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Conflict of interest

The authors declare that they have no conflict of interest in this publication.

REFERENCES

1. Akthar, M. F., S. Rashid, M. Ahmad, and K. Usmanghani. Cardiovascular evaluation of *Ruellia patula* and *Ruellia brittoniana*. *Journal of Islamic Academy of Sciences*, vol. 5, no. 1, pp. 67-71, 1992.
2. Anonymous. Wealth of India- A dictionary of Indian raw material and industrial products. National Institute of Science Communication, CSIR, New Delhi, India, vol. 5, pp. 360-364, 1959.
3. Agrawal, S. S. and V. K. Singh. Immunomodulators – A review of studies on Indian medicinal plant and synthetic peptides, Part – 1, Medicinal plants. *Proceedings of the Indian National Science Academy*, vol. 65, no. B, pp. 179-204, 1999.
4. Onayade, O. A., J. J. C. Scheffer, and A. B. Svendsen. The importance of phytotherapy and screening of plants used medicinally in Africa. *Planta Medica*, vol. 56, pp. 503-504, 1990.
5. Yoganandam, P., K. Sunil, V. Gopal, and K. Muthamizheselvam. Promising anti-leishmanial potentials of *Dipteracanthus prostrates* (Poir) Nees. in both axenic amastigotes and promastigotes of *Leishmania donovani*. *International Journal of Pharmacology and Toxicology*, vol. 4, pp. 19-23, 2014.
6. Chopra, R. N., S. L. Nasar, and I. C. Chopra. Glossary of Indian medicinal plants. *National Institute of Science Communication*, New Delhi, India, vol. 99, 1956.
7. Rastogi, R. P. and B. N. Mehrotra. Compendium of Indian medicinal plants. *Publications and Information Directorate*, CSIR, New Delhi, India, vol. 2, pp. 280, 1989..
8. Asolkar, L. V., K. K. Kakkar, and O. J. Chakre. Glossary of Indian medicinal plants with active principles. Part-1, *Publications and Information Directorate*, CSIR, New Delhi, India, pp. 280, 1992.
9. Palanisamy, P., B. Jayakar, M. V. Kumuthavalli, Y. Kumar, and K. R. Srinath. Preliminary phytochemical evaluation of whole plant extract of *Dipteracanthus prostrates* Nees. *International Research Journal of Pharmacy*, vol. 3, pp. 150-153, 2012.
10. Maruthupandian, A., V. R. Mohan, and Kottaimuthu Ethnomedicinal plants used for the treatment of diabetes and jaundice by palliyar tribals in sirumalai hills, Western Ghats, Tamil Nadu, India. *Indian Journal of Natural Products and Resources*, vol. 2, pp. 493-497, 2011.
11. Robert, J., B. R. Xavier, and C. Louis An efficient *in vitro* plant regeneration of *Dipteracanthus prostrates* (Poir.) Nees.- a medicinal herb. *Asian Pacific Journal of Tropical Biomedicine*, vol. 2, pp. 484-487, 2012.
12. Murashige, T., F. Skoog. A revised medium for rapid growth and bioassays with tobacco cultures. *Physiologia Plantarum*, vol. 15, pp. 473-497, 1962.
13. Shekhawat, M. S., N. S. Shekhawat, Harish, R. Kheta, M. Phulwaria, and A. K. Gupta. High frequency plantlet regeneration from nodal segment culture of female *Momordica dioica* (Roxb.). *Journal of Crop Science and Biotechnology*, vol. 14, no. 2, pp. 133-137, 2011.
14. Phulwaria, M., A. K. Patel, J. S. Rathore, K. Ram, and N. S. Shekhawat. An improved micropropagation and assessment of genetic stability of micropropgated *Salvadora oleoides* using RAPD and ISSR markers. *Acta Physiologiae Plantarum*, doi:10.1007/s11738-014-1486-z, 2014.
15. Lodha, D., A. Patel, and N. S. Shekhawat. A high-frequency *in vitro* multiplication, micromorphological studies and *ex vitro* rooting of *Cadaba fruticosa* (L.) Druce (Bahuguni): a multipurpose endangered medicinal shrub. *Physiology and Molecular Biology of Plants*, doi:10.1007/s12298-015-0310-6, 2015.
16. Kataria, V., N. S. Shekhawat. Cloning of *Rauvolfia serpentina* – an endangered medicinal plant. *Journal of Sustainable Forestry*, vol. 20, pp. 53-65, 2005.
17. Patel, A. K., M. Phulwaria, M. K. Rai, A. K. Gupta, S. khawat, and N. S. Shekhawat. *In vitro* propagation and *ex vitro* rooting of *Caralluma edulis* (Edgew.) Benth. & Hook. F.: An endemic and endangered edible plant species of the Thar Desert. *Scientia Horticulturae*, vol. 165, pp. 175-180, 2014.
18. Shekhawat, M. S., N. Kannan, M. Manokari. *In vitro* propagation of traditional medicinal and dye yielding plant *Morinda coreia* Buch. -Ham. *South African Journal of Botany*, doi:10.1016/j.sajb.2015.05.018, 2015.
19. Anand, S. P., E. Jayakumar, R. Jeyachandran, V. Nandagobalan, and A. Doss. Direct organogenesis of *Passiflora foetida* L. through nodal explants. *Plant Tissue Culture and Biotechnology*, vol. 22, no. 1, pp. 87-91, 2012.
20. Shekhawat, M. S., N. Kannan, M. Manokari, and C. P. Ravindran *In vitro* regernation of shoots and ex vitro rooting of an important medicinal plant *Passiflora foetida* L. through nodal segment cultures. *Journal of Genetic Engineering and Biotechnology*, doi:10.1016/j.jgeb.2015.08.002, 2015.
21. Panwar, D., K. Ram, Harish, and N. S. Shekhawat. *In vitro* propagation of *Eulophia nuda* Lindl., an endangered orchid. *Scientia Horticulturae*, vol. 139, pp. 46-52, 2012.
22. Rai, M. K., P. Asthana, V. S. Jaiswal, and U. Jaiswal. Biotechnological advances in guava (*Psidium guajava* L.). *Scientia Horticulturae*, vol. 21, pp. 1-12, 2010.
23. Pospsilovia, J., I. Ticha, P. Kadleck, D. Haisel, and S. Plzakova Acclimatization of micropropagated plants to *ex vitro* conditions. *Biologia Plantarum*, vol. 42, pp. 481-497, 1999.
24. Rathore, M. S. and N. S. Shekhawat. *In vitro* regeneration in *Sarcostemma acidum* (Roxb.) – an important medicinal plant of semi-arid ecosystem of Rajasthan, India. *Physiology and Molecular Biology of Plants*, vol. 19, pp. 269-275, 2012.