International Journal of Plant Developmental Biology ©2007 Global Science Books

Plant Regeneration from Leaf Explants of *Clematis gouriana* Roxb. – An Endemic Medicinal Plant of Western Ghats

H. Raja Naika • V. Krishna*

Plant Tissue Culture Laboratory, P.G. Department of Studies and Research in Biotechnology and Bioinformatics, School of Biological Sciences, Kuvempu University, Shankaraghatta – 577 451, Karnataka, India

Corresponding author: * **krishnabiotech2003@gmail.com**

ABSTRACT

Studies were undertaken to evaluate the regenerative capacity of leaf explants of *Clematis gouriana* Roxb. The leaf explants were excised from healthy plants and cultured on Murashige and Skoog medium (MS medium) variously supplemented with growth regulators. Regeneration occurred via organogenesis on MS medium supplemented with 3.5 to 5.5 mg l^{-1} 6-furfuryl aminopurine (FAP), 0.2 to 0.6 mg l^{-1} indole-3-butyric acid (IBA) and 0.2-0.6 mg l^{-1} thidiazuron (TDZ). The frequency of shoot bud production was the highest (mean of 10.90 ± 0.27 shoots per explants) at 4.5 mg l^{-1}, FAP 0.4 mg l^{-1} IBA and 0.4 mg l^{-1} TDZ. Regenerated shoots formed complete plantlets on medium containing 0.1 to 0.5 mg l^{-1} IBA as well as on MS basal medium without growth regulators. Matured plants were established, acclimatized, and thrived in greenhouse conditions and grew normally without any morphological variation. The regeneration protocol developed in this study provides a basis for germplasm conservation and for further investigation of medicinally active constituents of *C. gouriana*, a medicinal plant of Western Ghats.

Keywords: conservation, dehra, leaf culture, medicinal climber, organogenesis, Ranunculaceae
Abbreviations: **ANOVA**, analysis of variance; **BAP**, 6-benzylaminopurine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **FDD**, flora of Davanagere District; **FAP**, 6-furfurylaminopurine; **IBA**, indole-3-butyric acid; **NAA**, naphthaleneacetic acid; **RH**, relative humidity; **TDZ**, thidiazuron

INTRODUCTION

Clematis gouriana Roxb. (Ranunculaceae) is a woody climber (**Fig. 1**) very sparsely distributed in Western Ghats, India (Saldanha 1984). In the Indian system of medicine 'Ayurveda' the plant is used to eliminate malarial fever and headache. Root and stem paste is applied externally for psoriosis, itches and skin allergy (Nadakarni 1954). The traditional medicine practitioners residing in the vicinity of Bhadra Wild Life Sanctuary, India are using the leaf and stem juices for treating infectious old wounds, psoriasis, dermatitis, blood diseases, leprosy, wound healing, and cardiac disorders.

Phytochemically, the species of *Clematis* contains many secondary metabolites. Many investigators successfully isolated some of the secondary metabolites from the species of *Clematis*. Clemontanoside-C, a new hedragenin-based saponin isolate from the stem of *Clematis montana* (Thapliyal and Bahuguna 1993) and from the aerial part of *Clematis tibetana*, two new hederagenin, 28-*O*-bisdesmosides called clematibetosides A and C. A new gypsogenin 3, 28-*O*-bisdesmoside called clematibetoside B, were isolated together with ten known saponin (Kawata *et al*. 2001). Protoanmonin has been isolated from the Australian 'Headache Vine' *Clematis glycinoides* (Southwell and Tucker 1993).

Fig. 1 The *in vivo* plant of *Clematis gouriana* Roxb. showing leaves and flowers.

Since the harvest of medicinal plants on a mass scale from their natural habitats for extraction of bioactive compounds for commercial use is leading to a depletion of plant resources, the conservation of these valuable genotypes is imperative. In recent years, there has been an increased interest in *in vitro* culture techniques which offers a viable tool for mass multiplication and germplasm conservation of rare, endangered, and threatened medicinal plants (Li *et al*. 2004; Emma *et al*. 2005). Further, genetic improvement is another approach to augment the drug yielding capacity of the plants (Tejavathi and Shailaja 1999). The antimicrobial activity of different extracts were screened against twenty-seven clinical isolates from different infectious sources belonging to Gram-negative *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, and Gram-positive *Staphylococcus aureus* and five dermatitis fungi: *Trichophyton rubrum*, *T. tonsurans*, *Microsporum gypseum*, *M. audouini*, and *Candida albicans* has been reported from this species (Raja Naika and Krishna 2007) and *in vitro* micropropagation from nodal stem explants of *Clematis gouriana* has been reported (Raja Naika and Krishna 2007). Literature survey indicated that *in vitro* protocol was not yet standardized from leaf explant of this threatened climber. In view of its medicinal importance, threatening status, lack of tissue culture reports, the present study reports the prime protocol for regeneration from the leaf explants of *Clematis gouriana*.

Received: 25 June, 2007. Accepted: 8 August, 2007.

Original Research Paper

MATERIALS AND METHODS

Plant material and explant source

Tender twigs 3-4 cm long of *Clematis gouriana* were collected from a healthy plant growing in the University Medicinal plant garden located in Bhadra Wild Life Sanctuary, Karnataka, India. The plant was identified and authenticated by comparing with the authenticated specimen deposited at the Kuvempu University herbarium (Voucher specimen FDD 80). The twigs were thoroughly washed under running tap water for 25-30 min and then rinsed in a solution containing the surfactant Tween-20 (two drops in 100 ml solution) subsequently; they were surface-sterilized with 0.1% (w/v) $HgCl_2$ solution for 2-3 min, followed by three to five rinses with sterile distilled water in a clean air cabinet. The surface-sterilized leaves were aseptically cut into 2-4 mm segments and were carefully inoculated onto the culture media.

Culture media and culture conditions

The culture media consisted of MS salts (Murashige and Skoog 1962) augmented with 3% (w/v) sucrose and gelled with 0.8% (w/v) agar (Hi-Media, India) and various auxins, 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), and cytokinins, 6-benzylaminopurine (BAP), 6-furfurylaminopurine (FAP) at appropriate concentrations, both individually and in combinations (BAP with NAA, BAP with IAA, 2, 4-D with FAP, FAP with NAA and BAP with IBA). All plant growth regulators were added to the medium before autoclaving. The pH of the medium was adjusted to 5.6 to 5.8, autoclaved at 121°C at 15 psi (1.06 kg/cm^2) pressure for 15 to 40 min. Fifty ml medium was dispensed in sterilized 10.5 × 6.5 cm culture bottles (Varsha Storage Racks, Bangalore, India) closed with ebonite caps. The cultures were incubated at 28 ± 2°C and 60 µmol m^{-2} s^{-2} light intensity under 12 h photoperiod with cool-white fluorescent tubes (Philips, India) with 55% relative humidity.

In vitro rooting and acclimatization

For *in vitro* rooting, individual microshoots 6-8 cm long with 4 to 5 leaves were aseptically excised from the culture bottle and transferred to MS basal medium without growth regulators, as well as MS-semi solid medium supplemented with 0.1 to 0.5 mg l^{-1} IBA for root initiation. The rooted plants were removed from the culture bottles, washed with sterile distilled water and transferred to plastic pots with sterile vermiculite: perlite: peat moss (1:2:3 v/v/v) (Dugar Industries, India). The plantlets were placed in a growth chamber at 70% RH, 28 ± 2°C under a 12-h photoperiod for acclimatization. The plants were fertilized with 1/8th MS macronutrients twice during the course of acclimatization at an interval of 4-5 weeks. Established plants were placed in 20 cm diameter pots with sand: soil mixture (1:1) and transferred to a mist chamber (RH 80%; 34 ± 2°C) for hardening. These hardened plants were field-transferred and survival rate was recorded.

Statistical analysis

A minimum of 10 culture tubes were raised for each combination and all experiments were repeated 10 times. Analysis of Variance (ANOVA) and mean separations were carried out using Duncan's Multiple Range Test (DMRT, Gomez and Gomez 1984). Followed by Tukey's multiple comparison tests to assess the stastical significance. $P \leq 0.05$ was considered as statistically significant, using statistical software SPSS ver. 11 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

When leaf explants inoculated onto MS medium augmented with only BAP (0.5 to 2.5 mg l^{-1}), NAA (0.5-1.5 mg l^{-1}), IAA (0.5-1.5 mg l^{-1}), or 2,4-D (1.5 to 2.5 mg l^{-1}) only callus formed. However a combination of FAP and IBA supported shoot bud formation from leaf explant, higher concentration of BAP with lower concentraction of NAA also showed organogenic responses, but on further incubation they failed to develop into shoots. When the FAP with IBA showed the optimal organogenesis responses from the stem explant on 4.0 mg l^{-1} FAP and 0.5 mg l^{-1} IBA (Raja Naika and Krishna 2007). However FAP with IBA was also showed organogenic response from leaf explant on slightly increasing the FAP concentration, but when the TDZ was supplied at lower concentration the frequency of shoot bud formation was excellent, when compared to BAP with NAA and FAP with IBA supplemented medium. The formation of shoot buds occurred either on the adaxial or on the abaxial surface, whichever was in contact with the medium. The percentage frequency of explants forming shoot buds was the same (45-85%) irrespective of the orientation of the explant on the medium, Although shoot bud formation was observed in the concentrations of 3.5 to 5.5 mg l^{-1} FAP with 0.2 to 0.6 mg l^{-1} IBA, the number of buds produced per explant differed in each treatment. In *Campanula carpatica*, *Sesbania aculeata* from hypocotyl explant, *Celastrus paniculatus* from stem explant and *Embelia ribes* from leaf explant cytokinin (FAP) alone or in combination with lower concentration of auxins (IBA, NAA) provoked direct organogenesis from the explants (Bansal and Pandey 1993; Sriskandarajah *et al*. 2001; Maruthi *et al.* 2004; Shankarmurthy *et al*. 2004).

In the present study also synergistic effect of cytokinin and auxins induced adventitious shoot organogenesis from leaf explants. The combination of FAP with IBA at the range of 3.5 to 5.5 mg l^{-1}, 0.2 to 0.6 mg l^{-1} and lower concentration of TDZ respectively proved to be an optimal condition for adventitious organogenesis for shoot from the leaf explants. Within a week of incubation the explants became swollened, After 15 days of incubation, pale greenish photosynthetic protuberances were organized from the margin which later grew up into shoots without intervening the callus phase. Similar mode of organogenesis of the shoot buds was also reported for *Embelia ribes* on 3.0 mg l^{-1} FAP and 0.4 mg l^{-1} NAA (Shankarmurthy *et al*. 2004) for *Vitex negundo* using FAP, BAP and TDZ alone with 10% coconut milk (Nisha Rani and Nair 2006) and significantly more shoots (26 shoots per leaf explants) were induced on a medium containing 2.5 µM thidiazuron (TDZ) and 5.0 µM 1-naphthaleneacetic acid (NAA) in *Hydrastis canadensis* (He *et al*. 2007).

The effect of interaction of higher levels of FAP (3.5 to 5.5 mg l^{-1}) with lower levels of IBA (0.2 to 0.6 mg l^{-1}) and TDZ (0.2-0.6 mg l^{-1}) on adventitious shoot organogenesis is assessed in **Table 1**. However, the frequency of explants forming shoot buds decreased at (0.8 mg l^{-1}) IBA. This may be due to high endogenous auxin levels which lead to a decrease in the percentage response of explants to form shoot buds with an increase in the auxin concentration beyond a threshold value. Similarly, very young leaves fail to support adventitious shoot bud formation because they are the sites actively synthesizing auxins (Moore 1989). The caulogenic frequency was optimized at 4.5 mg l^{-1} FAP, 0.4 mg l^{-1} IBA and 0.4 mg l^{-1} TDZ. In four weeks old culture, shoot buds sprouted from the margin of the explant grew up well with large photosynthetic leaves. In addition, small photosynthetic protuberances arose all over the surface of the leaf explant which later developed into shoot buds (**Fig. 2A**). In six weeks old culture, a mean of 10.90 ± 0.27 shoots were counted per explant that grew up well into large photosynthetic leaves (**Fig. 2B**). The shoot buds organized directly from the explants through the leaf explant were harvested from the clump when they attained a length of more than 4-5 cm with 5-6 leaf primordia. They were transferred to basal medium without growth regulators and as well as MS-semi solid medium supplemented with 0.1 to 0.5 mg l^{-1} IBA showed root initiation from base of microshoots (**Fig. 2C**). Irrespective of their organogenic origin 90% of the shoots produced root initials from their base. However, the survival rate of the plantlets derived from direct organogenesis was 85%. The morphology of a month-old regenerants was similar with the *in vivo* plants (**Fig. 2D**).

The therapeutic properties of the medicinal plants are due to the presence of active principles, which has to be ex-

Table 1 Effect of FAP, IBA and TDZ on adventitious shoot bud induction and regeneration of plantlets through leaf explant culture of *Clematis gouriana*.

Growth regulators (mg l^{-1})			Number of shoot buds per explant	Number of rooted plantlets per explant
FAP	**IBA**	**TDZ**	**Mean ± SE**	**Mean ± SE**
3.5	0.2	0.2	1.20 ± 0.13 i	1.30 ± 0.15 ij
3.5	0.4	0.4	2.60 ± 0.22 h	1.50 ± 0.16 ij
3.5	0.6	0.6	4.00 ± 0.29 g	2.60 ± 0.16 h
4.0	0.2	0.2	6.70 ± 0.26 e	3.40 ± 0.22 g
4.0	0.4	0.4	7.10 ± 0.43d e	7.60 ± 0.16 d
4.0	0.6	0.6	9.90 ± 0.27 c	8.70 ± 0.16 c
4.5	0.2	0.2	11.00 ± 0.25 b	9.70 ± 0.36 b
4.5	**0.4**	**0.4**	**12.50 ± 0.37 a**	**10.90 ± 0.27 a**
4.5	0.6	0.6	11.40 ± 0.30 b	9.60 ± 0.33 b
5.0	0.2	0.2	9.70 ± 0.36 c	8.00 ± 0.25 d
5.0	0.4	0.4	7.90 ± 0.23 d	6.30 ± 0.21 e
5.0	0.6	0.6	7.30 ± 0.30d e	5.20 ± 0.29 f
5.5	0.2	0.2	5.10 ± 0.31 f	3.30 ± 0.21 g
5.5	0.4	0.4	3.80 ± 0.24 g	1.90 ± 0.23 i
5.5	0.6	0.6	2.10 ± 0.23 h	1.10 ± 0.17 j
F-value			**151.94**	**206.21**

In each column the mean value with different alphabetical letters are significantly different (P <0.05) according to DMRT test. The value of each concentration consisted of mean ± S.E. of 10 replicates.

tracted and screened for medicinal properties. Industrialization coupled with urbanization is constantly putting pressure on natural resources. Due to the depletion of habitat and ruthless collection pressure, medicinal plants are disappearing fast from the wild. Hence, conservation of germplasm becomes an urgent requirement. To circumvent further deterioration of the species in contrast *in vitro* culture offers a sustainable and viable tool for rapid propagation and storage of germplasm. The results of these studies have provided an efficient regeneration system for mass-propagation and extraction of secondary metabolites of *C. gouriana* for different purposes. Plantlets derived from leaf explants can provide masses of tissue for the biochemical characterization of medicinally active constituents, and selection and cloning of superior individual genotypes. This *in vitro* regeneration system may also be useful for crop improvement through genetic engineering and cell culture techniques. Together this approach may result in novel uses for this valuable species. Further work is under progress for the evaluation of phytochemical and pharmacological activities of *C. gouriana*.

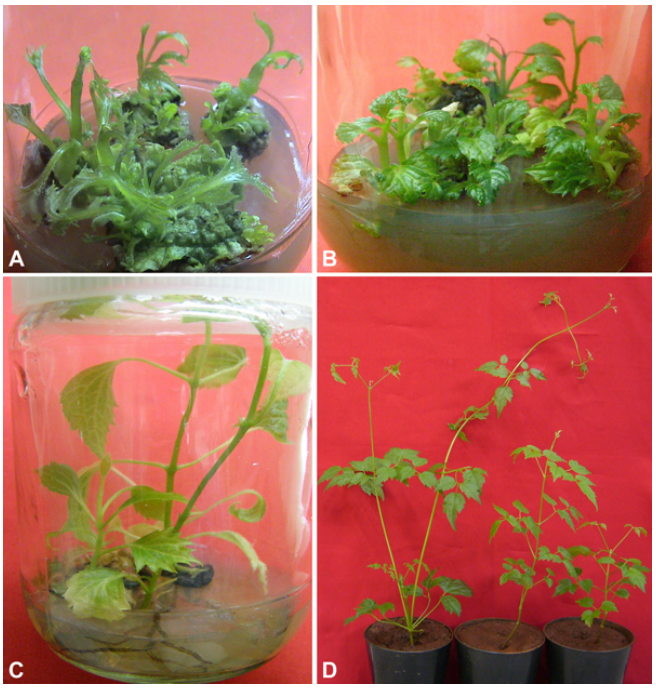

Fig. 2 (**A**) Adventitious shoot buds organized from the leaf explants on MS+4.5 mg l^{-1}, FAP 0.4 mg l^{-1} IBA and 0.4 mg l^{-1} TDZ. (**B**) Five weeks old culture showing shoot multiplication with 10.90 ± 0.27 shoots per explant. (**C**) Rhizogenesis from the micro shoots on MS + 0.5 mg l^{-1} IBA. (**D**). A month-old regenerants were acclimatized on sterile vermiculite: perlite: peat moss (1:2:3 v/v/v) with 70% RH, 28 ± 2°C.

ACKNOWLEDGEMENTS

The authors are thankful to The Registrar and Finance Officer, Kuvempu University. We acknowledge the support of Prof. Riaz Mahmood, Dept. of Biotechnology and Bioinformatics, Kuvempu University. We are also thankful to Mr. Kumara Swamy (SRF), Mr. Harish (SRF), Mr. Sharath (SRF) and Mr. Gouthamchandra (JRF) Kuvempu University, Shimoga, Karnataka.

REFERENCES

Bansal YK, Pandey P (1993) Micropropagation of *Sesbania acculeata* Pers. adventitious organogenesis. *Plant Cell, Tissue and Organ Culture* **32**, 351-355

Emma MJ, Sevdalina A, Verkleij ACJ (2005) Callus induction and plant regeneration in the metallophyte *Silene vulgaris* (Caryophyllaceae). *Plant Cell Tissue and Organ Culture* **80**, 25-31

Gomez KA, Gomez AA (1984) *Statistical Procedures for Agriculture Research*, John Willey and Sons, New York, USA, 680 pp

Kawata Y, Kizu H, Miyaichi Y, Tomimori T (2001) Studies on the constituents of *Clematis* species. VIII. Triterpenoid Saponins from the Aerial Part of *Clematis tibetana* Kuntz. *Chemical and Pharmaceutical Bulletin* **49**, 635-638

Li J, Jain M, Vunsh R, Vishnevetsky J, Hanaia U, Flaishman M, Perl A, Edelman M (2004) Callus induction and regeneration in *Spirodela* and *Lemna*. *Plant Cell Reports* **22**, 457-464

Maruthi KR, Krishna V, Nagaraja YP, Rahiman BA, Pullaiah T (2004) *In vitro* regeneration of *Celastrus paniculatus* Willd. – A rare medicinal plant. *Plant Cell Biotechnology and Molecular Biology* **5**, 33-38

Moore TC (1989) Auxins. In: Moore TC (Ed) *Biochemistry and Physiology of Plant Hormones* (2nd Edn), Springer-Verlag, Berlin, pp 28-93

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Nadakarni AKB (1954) *Indian Materia Medica*, Dhootapapeshwar. Prakashan Ltd., Mumbai, 452 pp

Nisha Rani D, Nair GM (2006) effects of plant growth regulators on high frequency shoot multiplication and callus regeneration of an important Indian medicinal plant, Nirgundi (*Vitex negundo* L). *In Vitro Cellular and Developmental Biology – Plant* **42**, 69-73

Raja Naika H, Krishna V (2007) Antimicrobial activity of extracts from the leaves of *Clematis gouriana* Roxb. *International Journal of Biomedical and Pharmaceutical Sciences* **1**, 69-72

Raja Naika H, Krishna V (2007) *In vitro* micropropagation of *Clematis gouriana* Roxb. from nodal stem explants. *The Asian and Australasian Journal of Plant Science and Biotechnology* **1**, 58-60

Saldanha CJ (1984) *Flora of Karnataka*, India, Oxford and IBH Publishing Co, New Delhi, pp 94-97

Shankarmurthy K, Krishna V, Maruthi KR, Rahiman BA (2004) Rapid adventitious organogenesis from leaf segments of *Embelia ribes* Burm. – A threatened medicinal plant. *Taiwania* **49**, 1-7

He SS, Liu CZ, Saxena PK (2007) Plant regeneration of an endangered medicinal plant *Hydrastis canadensis* L. *Scientia Horticulturae* **113**, 82-86

Southwell IA, Tucker DJ (1993) Protoanemonin in Australian *Clematis*. *Phytochemisry* **33**, 1099-1102

Sriskandarajah S, Frello S, Serek M (2001) Induction of adventitious shoots *in vitro* in *Campanula carpatica*. *Plant Cell, Tissue and Organ Culture* **67**, 295-298

Tejavathi DH, Shailaja KS (1999) Regeneration of plants from the cultures of *Bacopa monnieri* (L.) Pennell. *Phytomorphology* **49**, 447-452

Thapliyal RP, Bahuguna RP (1993) Clemontanoside-C, A saponin from *Clematis montana*. *Phytochemisry* **3**, 671-673