The Asian and Australasian Journal of Plant Science and Biotechnology ©2007 Global Science Books

In Vitro Micropropagation of *Clematis gouriana* Roxb. from Nodal Stem Explants

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ABSTRACT

A micropropagation protocol has been standardized for the medicinal climber *Clematis gouriana* Roxb. Adventitious shoots formed directly from nodal stem explants on Murashige and Skoog (MS) medium supplemented with 3.0 to 5.0 mg l^{-1} 6-furfuryl amino purine (FAP) and 0.3 to 0.7 mg l^{-1} indole-3-butyric acid (IBA). The frequency of shoot bud production was the highest (mean of 11.80 ± 0.32 shoots per explant) at 4.0 mg l^{-1} FAP and 0.5 mg l^{-1} IBA. The microshoots rooted well on MS basal medium without plant growth regulators, as well as on MS medium supplemented with 0.1 to 0.5 mg l^{-1} IBA. Regenerated plants were successfully acclimatized and about 80% of the hardened regenerants survived under natural conditions.

Keywords: dehra, medicinal climber, organogenesis, stem culture
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**, α-naphthalene acetic acid; **RH**, relative humidity

INTRODUCTION

Clematis gouriana Roxb. (Ranunculaceae) is a woody climber (**Fig. 1**) distributed in Western Ghats, India (Saldanha 1984). In the Indian system of medicine 'Ayurveda' the plant is used to alleviate malarial fever and headache. Root and stem paste is applied externally for psoriosis, itches and skin allergy (Manjunatha *et al*. 2004). 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.

Plants are valuable sources of a vast array of chemical compounds; they synthesize and accumulate extractable organic substances in quantities sufficient to be economically useful raw materials for various commercial applications. Phytochemically, *Clematis* spp. contain many secondary metabolites from which many investigators successfully isolated some secondary metabolites. Clemontanoside-C, a new hedragenin-based saponin isolate from the stem of *Clematis montana* (Thapliyal and Bahuguna 1993) and from the aerial part (stem, leaves and flowers) 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 saponins (Kawata *et al*. 2001). Protoanmonin has been isolated from the Australian 'Headache Vine' *Clematis glycinoides* (Southwell and Tucker 1993).

Fig. 1 ***Clematis gouriana*** **Roxb. plant showing leaves and flowers.**

Owing to indiscriminate over-exploitation for medicinal purposes and destruction of the natural habitat by anthropogenic activities there is an alarming reduction of the population in the wild. As a result of these factors, many medicinal plants have become threatened species. *In vitro* technique is a promising tool for *ex situ* conservation of germplasm of valuable medicinal herbs that can be used for the extraction of active compounds for commercial use (Krogstrup *et al.* 1992). There are sufficient reports available about the protocols on *in vitro* micropropagation of many threatened medicinal species (Emma *et al*. 2005). A literature survey indicated that an *in vitro* protocol has not yet been standardized for this rare climber. In view of its medicinal importance and threatening status *in vitro* studies were undertaken to regenerate and *C. gouriana* plantlets directly through shoot multiplication using stems as initial explants and plantlets could be rapidly multiplied via adventitious organogenesis. The present paper has great relevance from a conservation point of view.

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.

Received: 25 June, 2007. Accepted: 27 July, 2007.

Short Communication

The plant was identified 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 3-5 rinses with sterile distilled water in a clean air cabinet. The surface-sterilized explants were trimmed to 0.5-1.5 cm in length, comprising a single node each. The explants were carefully inoculated randomly onto callogenic and caulogenic 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-dichloro-phenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA), and cytokinins, 6-benzylaminopurine (BAP), 6-furfurylaminopurine (FAP) at specific concentrations, both individually and in combinations (BAP with NAA, FAP with IBA, 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. A quantity of 50 ml medium was dispensed in sterilized 10.5 × 6.5 cm culture bottles (Varsha Storage Racks, Bangalore, India) closed with ebonite caps, each culture bottle containing four explants. The cultures were incubated at 28 ± 2°C and 60 µmol m^{-2} s^{-2} light intensity under a 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 transferred to the field and the 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). The nature and percentage of response were recorded at an interval of one week. Sub culturing was periodically carried out at 4-week intervals.

RESULTS AND DISCUSSION

The stem explants inoculated onto MS medium augmented BAP with lower concentration of auxins NAA, IAA, IBA and 2, 4-D with FAP, only the callus formation was noticed from the explants. MS medium augmented with 3.0 to 5.0 mg l^{-1} FAP showed an organogenic response. The explants retained their photosynthetic activity and enlarged to three times their initial size. At 2.0 mg l^{-1} FAP, two to three shoot buds organized directly from the explant but on further incubation they failed to develop into shoots. 2,4-D at 1.5 to 2.5 mg l^{-1} initiated only callus from explants (**Fig. 2**). The

Fig. 2 Callus mass developed on 2,4-D at 1.5 to 2.5 mg l^{-1}.

Fig. 3 Initiation of pale green photosynthetic protuberances from the stem explants.

type and concentration of cytokinin has an immense effect on shoot bud organogenesis. Among different concentrations of BAP and FAP tested, adventitious shoot organogenesis was noticed only on FAP-supplemented medium whereas BAP at low concentrations (0.5 to 1.5 mg l^{-1}) stimulated the cells to form a callus mass. In *Campanula carpatica*, *Sesbania aculeata* from hypocotyl explant and *Embelia ribes* from leaf explant cytokinin alone or in combination with lower concentration of auxins provoked direct organogenesis from explants (Bansal and Pandey 1993; Sriskandarajah *et al.* 2001; Shankarmurthy *et al*. 2004). In the present study also the synergetic effect of cytokinin and auxins induced adventitious shoot organogenesis from the stem explant. The combination of FAP with IBA at the range of 3.0 to 5.0 mg l^{-1} and 0.3 to 0.7 mg l^{-1}, respectively proved to be optimal for adventitious organogenesis, i.e. shoot formation from stem explants. Within a week of incubation the explants became swollen; After 15 days of incubation, pale green photosynthetic protuberances organized from the margin which later grew into shoots without an intervening the callus phase (**Fig. 3**). A similar mode of organogenesis of the shoot buds was also reported on *Coffea bengalensis* (Mishra and Sreenath 2003).

The effect of interaction of higher levels of FAP (3.0 to 5.0 mg l^{-1}) with lower levels of IBA (0.3 to 0.7 mg l^{-1}) on adventitious shoot organogenesis is summarized in **Table 1**. An increase in the concentration of IBA (above 0.8 mg l^{-1}) and FAP (above 5.0 mg l^{-1}) inhibited the shoot organogenic potential of stem explants. One of the possible roles of a higher concentration of auxins in the organogenic stage is to nullify the effects of cytokinins on shoot organogenesis and elongation. Caulogenic frequency was optimal at 4.0 mg l^{-1} FAP and 0.5 mg l^{-1} IBA. In four-weeks old culture, shoot buds sprouted from the margin of the explant and developed

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

Growth regulators (mg l^{-1})		Number of shoot buds/explant	Number of rooted plantlets/explant
FAP	**IBA**	**Mean ± SE**	**Mean ± SE**
3.0	0.3	1.80 ± 0.20 h	1.50 ± 0.22 hi
3.0	0.5	3.40 ± 0.22 fg	1.70 ± 0.21 hi
3.0	0.7	4.40 ± 0.37 ef	3.10 ± 0.23 g
3.5	0.3	7.30 ± 0.42 d	3.60 ± 0.26 g
3.5	0.5	7.90 ± 0.52 d	7.90 ± 0.23 d
3.5	0.7	10.60 ± 0.45 c	9.10 ± 0.40 c
4.0	0.3	11.90 ± 0.34 b	10.20 ± 0.41 b
4.0	**0.5**	**13.00 ± 0.44 a**	**11.80 ± 0.32 a**
4.0	0.7	12.40 ± 0.37 ab	9.90 ± 0.34 b
4.5	0.3	9.70 ± 0.36 c	8.50 ± 0.34 cd
4.5	0.5	8.30 ± 0.30 d	6.90 ± 0.23 e
4.5	0.7	7.80 ± 0.38 d	5.90 ± 0.27 f
5.0	0.3	5.30 ± 0.30 e	3.80 ± 0.20 g
5.0	0.5	3.90 ± 0.23 fg	2.20 ± 0.24 h
5.0	0.7	2.90 ± 0.23 g	1.20 ± 0.13 i
F-value		104.14	160.73

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.

Fig. 4 (**A**) Adventitious shoot buds organized from the stem explants on MS + 4.0 mg l^{-1} FAP and 0.5 mg l^{-1} IBA. (**B**) Five weeks-old culture showing shoot multiplication with 11.80 ± 0.32 shoots per explant. (**C**) Rhizogenesis from shoots on MS + 0.5 mg l^{-1} IBA. (**D**) Eight weeks-old regenerants acclimatized on sterile vermiculite: perlite: peat moss (1:2:3 v/v/v) with 70% RH, 28 ± 2°C.

well forming large photosynthetic leaves. In addition, small photosynthetic protuberances arose all over the surface of the explant which later developed into shoot buds (**Fig. 4A**). In six-weeks old culture, a mean of 11.80 ± 0.32 shoots were counted per explant, and these also developed well into large photosynthetic leaves (**Fig. 4B**). The shoot buds organized directly from the explants through the stem explant were harvested from the clump when they attained a length of more than 5 cm with 5-6 leaf primordia. They were transferred to basal medium without growth regulators containing 0.8% (w/v) agar or to MS semi-solid medium containing a low concentration of agar 0.45% (w/v) supplemented with 0.1 to 0.5 mg l^{-1} IBA initiated roots from the base of microshoots (**Fig. 4C**). 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 80%. The morphology of one month-old regenerants was similar to the *in vivo* plants (**Fig. 4D**).

The therapeutic properties of medicinal plants are due to the presence of active principles, which have to be extracted 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 protocol reported here could be used for the *ex situ* conservation of this endemic and threatened medicinal plant and extraction of secondary metabolites from *C. gouriana*.

ACKNOWLEDGEMENTS

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

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