Turkish Journal of Biology

Volume 32 | Number 2 Article 5

1-1-2008

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HANUMANAIKA RAJA NAIKA

VENKATARANGAIAH KRISHNA

Turk J Biol
32 (2008) 99-103

Plant Regeneration from Callus Culture of *Clematis gouriana* Roxb. – A Rare Medicinal Plant

Hanumanaika RAJA NAIKA, Venkatarangaiah KRISHNA

Plant Tissue Culture Laboratory, Department of P.G. Studies and Research in Biotechnology and Bioinformatics,

School of Biological Sciences, Kuvempu University, Shankaraghatta - 577 451, Karnataka - INDIA

Received: 21.11.2007

Abstract: An in vitro regeneration protocol through stem callus culture has been standardized for the medicinal climber *Clematis gouriana*. The explant induced callus on MS-medium supplemented with 0.5 to 1.5 mg l^{-1} BAP and 0.1 to 0.5 mg l^{-1} NAA. The optimized callus induction occurred at the concentration of 1.0 mg l^{-1} BAP and 0.3 mg l^{-1} NAA. After initiation of callus, it was immediately transferred to MS medium containing 4.0 mg l^{-1} FAP and 0.5 mg l^{-1} indole-3-butyric acid (IBA). Upon longer incubation for about 2-6 weeks on the same culture medium initiation of shoot buds from the callus mass was observed and regeneration of plantlets with higher frequency (mean of 11.1 ± 0.23 shoots per explants) was noted. The microshoots rooted well on MS basal medium without growth regulators, as well as on medium supplemented with 0.5 mg l^{-1} IBA. Regenerated shoots formed complete plantlets on medium containing 0.5 mg l^{-1} IBA, and mature plants were established, acclimatized, and thrived in greenhouse conditions. The regeneration protocol developed in this study provides a basis for germplasm conservation and for further investigation of bioactive constituents of this medicinal plant.

Key Words: Clematis gouriana, medicinal climber, stem explant, plant regeneration

Nadir Bulunan Bir Tıbbi Bitki Olan *Clematis gouriana* Roxb.'un Kallus Kültüründen Bitki Rejenerasyonu

Özet: Sarmaşık türü olan *Clematis gouriana* tıbbi bitkisinin gövde kallus kültürü in vitro rejenerasyon çalışmaları için standardize edilmiştir. Bitkiden alınarak kültür ortamına yerleştirilen eksplantlarda, 0,5 'den 1,5 mg l^{-1} BAP ve 0,1 'den 0,5 mg l^{-1} NAA ilave edilen MS-besiyerinde kallus oluşumu çalışılmıştır. Kallus oluşumu 1,0 mg l^{-1} BAP ve 0,3 mg l^{-1} NAA konsantrasyonda optimize edilmiştir. Kallus oluştuktan hemen sonra FAP ve 0,5 mg l^{-1} indol-3-butirik asit (IBA) içeren MS-besiyerine aktarılmıştır.Yaklaşık 2-6 hafta süre aynı besiyerinde inkübe edilen kallus kütlesinden filizlenen tomurcuklar gözlemlenmiş ve daha yüksek frekanslı (her bir eksplant için filizler 11,1 ± 0,23 olması) bitkiciklerin rejenerasyonuna dikkat edilmiştir. Küçük filizler, hem MS temel besiyerinde hem de 0,5 mg l^{-1} IBA ilave edilen besiyerinde büyüme düzenleyicileri olmadan iyi bir şekilde gelişmiştir. Yeniden üretilen filizler 0,5 mg l^{-1} IBA içeren besiyerinde bütün bitkicikleri oluşturmuş ve olgunlaşan bitkiler tespit edilmiş, iklim koşullarına alıştırılmış ve sera koşullarında büyütülmüştür. Bu çalışmada geliştirilen rejenerasyon protokolü bitki üreme hücrelerinin korunması ve bu tıbbi bitkinin biyoaktif bileşenlerinin daha ileriki çalışmalarda araştırılması için bir temel oluşturmaktadır.

Anahtar Sözcükler: Clematis gouriana, tıbbi sarmaşık, eksplant, bitki rejenerasyonu

Abbreviations: ANOVA: analysis of variance; BAP: 6-benzylaminopurine; 2, 4-D: 2, 4-dichlorophenoxyacetic acid; FDD: flora of Davanagere District; FAP: 6-furfuryl aminopurine; IBA: indole-3-butyric acid; NAA: naphthalene acetic acid; MS: Murashige and Skoog; RH: relative humidity.

Introduction

Plants are a valuable source of a vast array of chemical compounds; they synthesize and accumulate extractable organic substances in quantities sufficient to be economically useful as raw materials for various commercial applications. Industrialization coupled with urbanization is constantly putting pressure on natural resources. Due to depletion of habitat and ruthless collection, medicinal plants are on the verge of extinction. Hence, the conservation of these valuable genotypes is imperative. Plant tissue culture technology holds great promise for micropropagation, conservation, and

enhancement of the natural levels of valuable secondary plant products and to meet pharmaceutical demands and reduce the in situ harvesting of natural forest resources. For mass propagation of medicinal plant species in which conventional methods possess limitations, in vitro multiplication provides the way out. There are sufficient reports available about protocols on in vitro micropropagation of many threatened medicinal species (1,2).

Clematis gouriana Roxb. (Ranunculaceae) is a woody climber very sparsely distributed in Western Ghats, India (3). 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 psoriasis, itches, and skin allergies. The traditional medicine practitioners residing in the vicinity of Bhadra Wildlife Sanctuary, India, use the leaf and stem juices for treating infected wounds, psoriasis, dermatitis, blood diseases, leprosy, and cardiac disorders (4). Phytochemically, the species of *Clematis* contain many secondary metabolites. Many scientific investigators successfully isolated some of the secondary metabolites from the species of *Clematis*. Clemontanoside-C, a new hedragenin-based saponin was isolated from the stem of *Clematis montana* (5) and from the aerial parts of *Clematis tibetana* 2 new hederagenin, 28-*O*-bisdesmosides called clematibetosides A and C. A new gypsogenin 3,28-*O*-bisdesmoside called clematibetoside B was isolated together with 10 known saponins (6). Protoanemonin has been isolated from the Australian 'Headache Vine' *Clematis glycinoides* (7).

As 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 increased interest in in vitro culture techniques, which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered, and threatened medicinal plants (8,9). Further, genetic improvement is another approach to augment the drug yielding capacity of plants (10). The antimicrobial activity of different extracts was screened against 27 clinical isolates from various different infectious sources, which included Gram-negative *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, Gram-positive *Staphylococcus aureus* and 5 dermatitis fungi: *Trichophyton rubrum*, *T. tonsurans*, *Microsporum gypseum*, *M. audouini*, and *Candida albicans* (11). A literature survey indicated that the in vitro protocol for stem callus culture of this threatened climber was not yet standardized. In view of its medicinal importance and threatened status, and the lack of tissue culture reports, the present study reports the prime protocol for regeneration from stem callus culture of *Clematis gouriana.*

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 Wildlife Sanctuary, Karnataka, India. The plant was identified and authenticated by comparing it 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 (2 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 to 5 rinses with sterile distilled water in a clean air cabinet. The surface-sterilized explants were aseptically cut into 2-4 cm segments and were carefully inoculated onto the culture media.

Culture media and culture conditions

The culture media consisted of MS salts (12) 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 as well as in combinations (BAP with NAA, BAP with IAA, 2, 4-D with FAP, FAP with NAA, and FAP 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, followed by autoclaving at 121 °C at 15 psi (1.06 kg/cm^2) pressure for 15 to 40 min. Fifty milliliters of 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) and 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 1/8 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 a 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 performed 10 times. Analysis of variance (ANOVA) and mean separations were carried out using Duncan's multiple range test, followed by Tukey's multiple comparison tests to assess the statistical significance. $P \leq 0.05$ was considered to be statistically significant, using statistical software SPSS ver. 11 (SPSS Inc., Chicago, USA).

Results and Discussion

For the induction of callus and multiple shoots from the stem explants of *Clematis gouriana*, explants were inoculated on MS medium supplemented with various concentrations of auxins and cytokinins. Callus or callusing of *Clematis gouriana* was observed from the explants on MS medium containing combinations of BAP, NAA, and 2,4-D. Callus was induced on MS medium fortified with 0.5 to 1.5 mg l^{-1} BAP and 0.1 to 0.5 mg l^{-1} NAA, as well as on 2,4-D at the concentration of 1.5 to 2.5 mg l^{-1}, only the callus was induced from the explant. Callusing of *Clematis gouriana* was optimized at the concentration of 1.0 mg l^{-1} BAP and 0.3 mg l^{-1} NAA, but the callus obtained from 2,4-D supplemented medium failed to produce shoot buds, whereas BAP and NAA supplemented medium showed signs of callogenic response and produced callus from the explant. A similar mode of response was observed in the species of *Valeriana edulis* (13) *Dioscorea zingiberensis* (14), and *Clerodendrum serratum* (15).

The callus was subcultured onto MS medium supplemented with various concentrations and combinations of auxins and cytokinins with BAP + NAA, FAP + IBA, and 2,4-D + FAP. Among these combinations BAP with NAA showed an organogenic response and produced only 2 to 3 shoot buds and with 2,4-D + FAP combinations only callusing was noted, whereas in FAP + IBA combinations there was an organogenic response and shoot buds were produced from the callus. MS medium containing 3.5-4.5 mg l^{-1} FAP and 0.1-0.7 mg l^{-1} IBA showed the organogenic responses depicted in the Table, and at 4.0 mg l^{-1} FAP and 0.5 mg l^{-1} IBA shoot buds arose all over the callus mass (Figure 1A). Upon incubation for 2-6 weeks on the same concentration medium plantlets

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

Growth regulators (mg l^{-1})		Number of shoot buds per explant	Number of rooted plantlets per explant
FAP	IBA	Mean ± SE	Mean ± SE
3.5	0.3	1.0 ± 0.00 j	1.0 ± 0.00 j
3.5	0.4	1.4 ± 0.16 ij	1.4 ± 0.16 ij
3.5	0.5	2.2 ± 0.24 gh	3.0 ± 0.29 g
3.5	0.6	2.9 ± 0.31 g	7.1 ± 0.27 d
3.5	0.7	7.1 ± 0.27 d	8.2 ± 0.24 c
4.0	0.3	8.1 ± 0.27 c	8.2 ± 0.24 c
4.0	0.4	9.3 ± 0.36 b	9.4 ± 0.37 b
4.0	0.5	12.2 ± 0.29 a	11.1 ± 0.23 a
4.0	0.6	9.7 ± 0.30 b	9.7 ± 0.30 b
4.0	0.7	7.5 ± 0.30 cd	7.6± 0.26 cd
4.5	0.3	5.9 ± 0.31 e	5.9 ± 0.31 e
4.5	0.4	4.8 ± 0.29 f	4.8 ± 0.29 f
4.5	0.5	2.4 ± 0.16 gh	2.4 ± 0.16 gh
4.5	0.6	1.9 ± 0.23 hi	1.9 ± 0.23 hi
4.5	0.7	0.8 ± 0.13 j	0.8 ± 0.13 j
F value:		196.21	196.65

In each column the mean values 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.

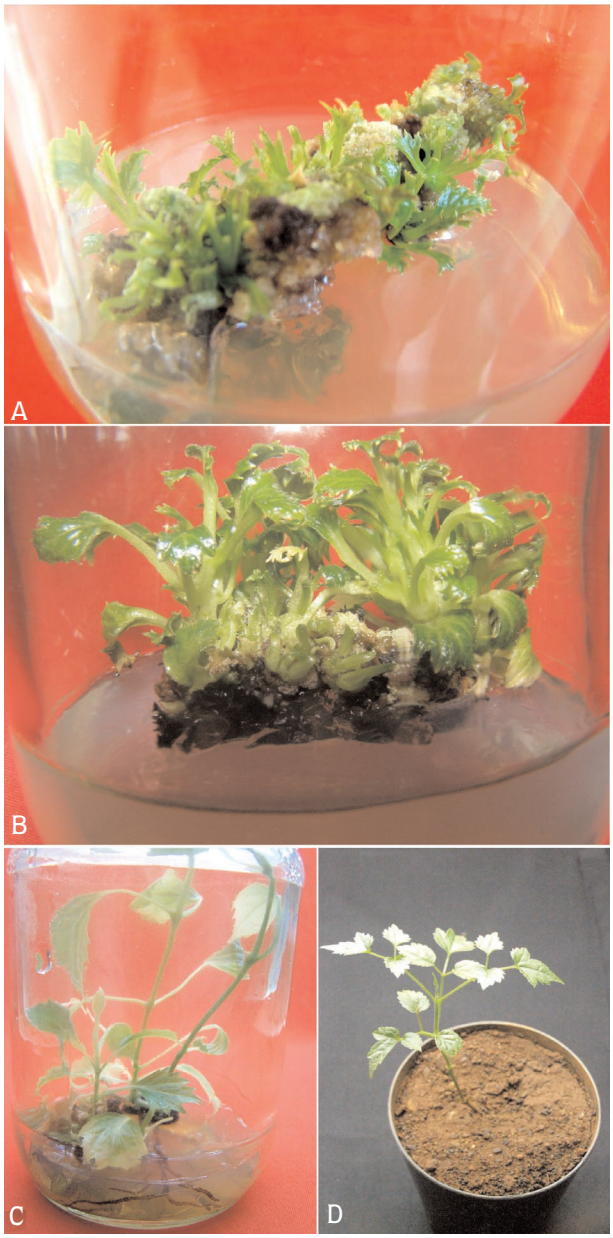

Figure 1. A. Shoot buds arose all over the callus mass at 4.0 mg l^{-1} FAP, 0.5 mg l^{-1} IBA.
B. Upon incubation for 2-6 weeks on the same medium, the plantlets arose all over the callus mass and a mean of 11.1 ± 0.23 shoots per explants were recovered.
C. 0.5 mg l^{-1} IBA pretreated microshoots showing profuse root system and establishment of root intact plantlets.
D. Hardened and soil acclimatized regenerants.

arose all over the callus mass and a mean of 11.1 ± 0.23 shoots per explants (Figure 1B) was recovered and this concentration was considered optimal. *Celastrus paniculatus* (16) and *Embelia ribes* (17) also showed regeneration of shoot buds from the callus mass with FAP and IBA combinations.

The microshoots were harvested from the clump when they attained a length of more than 4-5 cm with 3-4 leaf primordia. They were transferred to basal medium without growth regulators containing 0.8% (w/v) agar or MS semi-solid medium containing a low concentration of agar 0.45% (w/v) supplemented with 0.5 mg l^{-1} IBA initiated roots from the base of microshoots (Figure 1C). In *Naravelia zeylanica* a similar mode of rooting was reported (18). The rooted plantlets were subjected to a hardening process and were established, acclimatized, and thrived in greenhouse conditions (Figure 1D).

Tissue culture technology offers an alternative method for the conservation of germplasm as well as micropropagation of medicinally important plant resources. Presently there is great demand for the use of plant based medicaments in place of synthetic drugs. As a result of non-scientific exploitation, most of the medicinal plant resources are being threatened and are on the verge of extinction. Therefore, application of this technology provides the raw materials required for the isolation of drugs by the pharmaceutical industries without depleting natural plant resources.

Acknowledgements

The authors are grateful to the University Grant Commission, New Delhi, for providing financial aid. We acknowledge the support of Prof. Riaz Mahmood and Dr. Manjunatha H, Dept. of Biotechnology and Bioinformatics, Kuvempu University. We are also grateful to Mr. Pramod Kumar, Mr. Kumara Swamy (SRF), Mr. Harish (SRF), Mr. Sharath (SRF) Mr. Gouthamchandra (JRF), and Mr. Joy. H. Hoskeri, Kuvempu University, Shimoga, Karnataka.

Correspondence author:
Venkatarangaiah KRISHNA
Department of Biotechnology and Bioinformatics
School of Biological Sciences,
Kuvempu University,
Shankaraghatta - 577 451, Karnataka - INDIA
E – mail: krishnabiotech2003@gmail.com

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