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In vitro cell culture of *Charybdis congesta* for enhanced production of secondary metabolites: Proscillaridin A, Scillaren A and Scilliroside

A. Shiva Reddy[1], P. Sita Devi[1] and S. Ravi Kiran[2]*

[1]Analytical Chemistry Division, Indian Institute of Chemical Technology, Hyderabad – 500007, India.
[2]Department of Biochemistry and Biotechnology, Aurora's Degree & PG College, Chikkadpally, Hyderabad – 500020, India.

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Callus cultures of *Charybdis congesta* were initiated *in vitro* and the effect of growth regulators was tested on callus growth and secondary metabolite production. Among several standard media formulated for use in the present study, MS and B5 were found to be potentially active and facilitated the calculation of callus induction frequency (CIF). The CIF was higher in both MS (70%) and B5 (63%) media supplemented with 1-naphthalene acetic acid (NAA) (9.0 µM) and benzyl amino purine (BAP) (0.9 µM). However, with indole-3-butyric acid (IBA) (9.0 µM) and BAP (0.8 µM), less amount (22.6%) of CIF was observed in MS medium but no callus formation was noticed in B5 medium. Rapid high performance thin layer chromatography (HPTLC) screening of callus extracts revealed that the callus established in MS medium supplemented with 4.5 µM NAA and 0.46 µM BAP produced the highest yield of Proscillaridin A (4.51 mg/g DW), Scilliroside (3.3 mg/g DW), Scillaren A (2.35 mg/g DW) and desacetylscilliroside (8.62 mg/g DW), which was higher than from the intact plants. The results obtained indicate that the *in vitro* cultures of *C. congesta* might be an excellent source of secondary metabolites and further metabolic profiling may provide insights into up scaling of the compounds which lead to greater commercial interest and continuous supply of cultures.

Key words: Squill, *Charybdis congesta*, bufadienolides, callus cultures, reflectance scanning densitometry.

INTRODUCTION

Charybdis congesta, formerly known as *Urginea congesta*, belonging to the family Hyacinthaceae is a perennial bulbous species found in dry sandy places and is commonly known as Indian Squill. The bulbs of Squill contain various steroidal glycosides known as bufadienolides (0.15 to 2.4%) which include Scillarin A (SA), Scilliroside (SC), Scilliglauside (SG), Proscillaridin A (PA), Desacetylscillirosidine (DAS), and Scillirosidine (SDN) (Kopp et al., 1996), possessing interesting medicinal, rodenticidal and insect repellent properties (Verbiscar et al., 1986; FAIR961436, 1999; Pascual-Villalobos, 2002). At present, the most exploited source of these bufadienolides is bulbs of *C. congesta* and often requires large amounts of plant material which makes the plant endangered because of over-harvesting (Babu et al., 2001; Namdeo, 2007). Further, the conventional methods of propagation suffer from various limitations such as non-availability of plants on time due to geographical and unforeseen environmental conditions. Therefore, there is a need for effective and alternative sources for the propagation and production of

*Corresponding author. E-mail: rksuripeddi@yahoo.co.in. Tel: +91-40-27662668.

Figure 1. *C. congesta* bulb collected from Osmania University, Hyderabad, India.

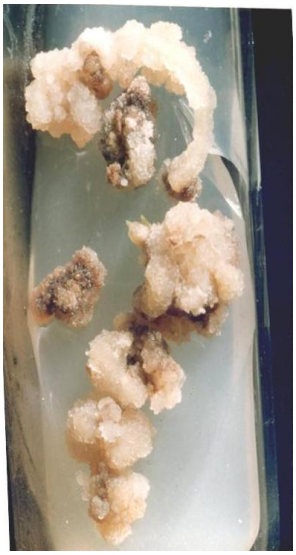

Figure 2. Callus obtained from bulbs as explants cultured on MS medium.

secondary metabolites. In this connection, biotechnological approaches, specifically, plant tissue culture becomes a powerful tool for mass multiplication and is found to have right potential for the production of many secondary metabolites from elite and threatened species (Dicosmo and Misawa, 1995; Thanonkeo and Panichajakul, 2005; Liu et al., 2006, Roostika et al., 2007). Cell cultures often have a higher rate of metabolism than intact plants due to fast proliferation and condensed biosynthetic cycle which makes them model systems for the study of secondary metabolite production as well as micropropagation. The tissue culture technique has been developed for many species of the family (Jha et al., 1984, 1991; Garari and Backhauss, 1987; Wawrosch et al., 2005) but *C. congesta* which is a South Indian variety, has not been so far studied and to the best of our knowledge, there are no published reports. Therefore, the aim of the present study was to develop a methodology for *in vitro* culture of *C. congesta*, extract bufadienolides from callus and evaluate the results to see how quantitative the present studies are with respect to tissue culture programme and the potential utility of the same. Here in, the investigative study carried out is described and the results are discussed.

MATERIALS AND METHODS

Bulbs of *C. congesta* (Figure 1) were procured from Osmania University, Hyderabad, India. A voucher specimen has been deposited at the Department of Botany, Osmania University, Hyderabad, India (No. OU BOT 4964). Reference Standard of Proscillaridin A, Scilliroside, desacetylscilliroside, Scillaren A, solvents and reagents were purchased from Merck chemicals, India.

Callus induction

To establish callus cultures, the bulbs cut into small explants consisting of two scale segments attached to a portion of the basal plate were used. Explants were inoculated individually into various media supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar and various combinations of growth regulators, auxin (1.5, 3.0, 4.5, 6.0 and 9.0 µM 2,4-D, NAA and IAA) and cytokinins (0.25, 0.45, 0.75 and 0.95 µM BAP and Kinetin). The cultures were kept under a 16 h photoperiod of 40 µmol $m^{-2}s^{-1}$ (Osram Biolux tubes) at 25 ± 1°C. Subcultures were routinely carried out every two weeks and all experiments were performed in quintuplicate. The suitable phytohormone combination was selected based on the more CIF obtained.

Measurement of callus growth

The cultures were scored for their ability to produce callus in terms of callus induction frequency (CIF) at the end of four to six weeks. The frequency was calculated as the ratio of number of explants responding to the total number of explants inoculated and expressed in percentage. Subsequently, growth measurement of callus was measured as dry weight (DW) by harvesting the biomass and drying at 60°C in a hot air oven to a constant weight.

Statistical analysis

All the experiments were performed in quintuplicate and the significance of the results obtained was verified by using Statistical Analysis Software (SAS®, 2000) and the data were presented as mean ± standard error (SE).

Extraction of PA, SC and SA from bulbs and callus cultures

Callus (Figure 2) obtained at the end of 7th week in MS (Murashige and Skoog 1962) and B5 (Gamborg et al., 1968) media supplemented with NAA and BAP was selected for extraction and quantification of active compounds. The callus was powdered and extracted each time with methanol, chloroform: methanol (8:2), chloroform: ethanol (8:2) and chloroform. The solution was subsequently

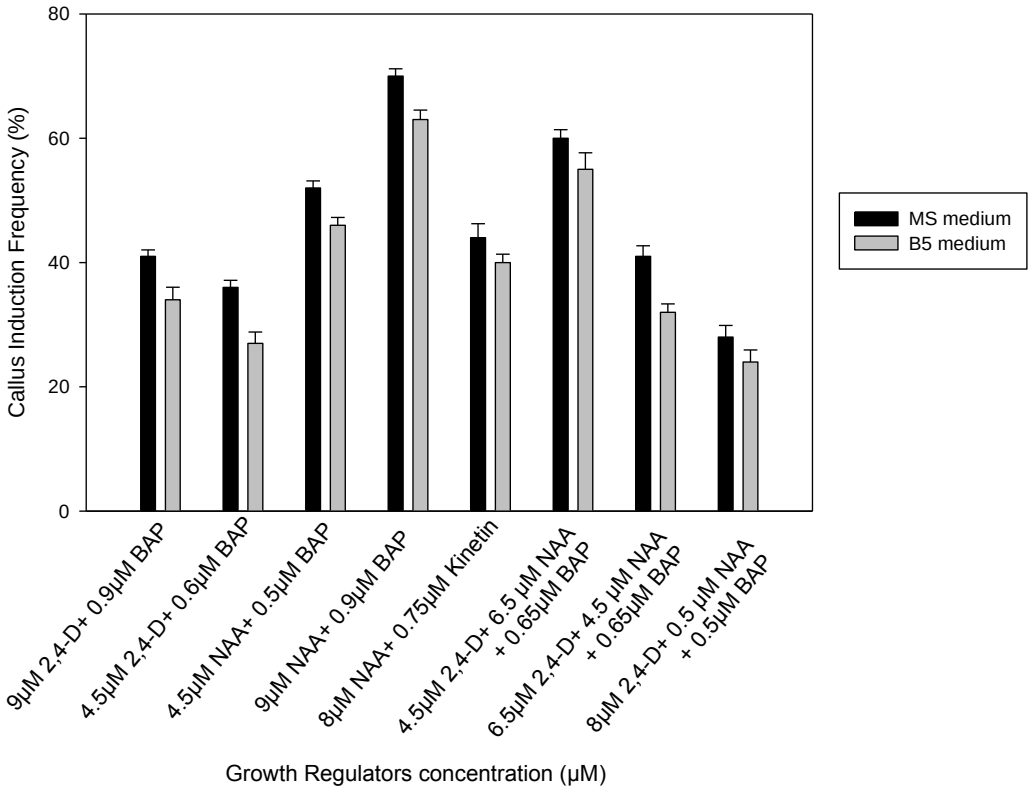

Figure 3. Callus induction frequency (%) of bulb explants cultured on MS and B5 media at different concentrations of growth regulators.

filtered and excess solvent was evaporated under vacuum in a rotary evaporator. Similarly, the crude extracts were prepared using bulbs as such by the same procedure described. The samples were assayed by high performance thin layer chromatography for their identity and quantification of active compounds.

High performance thin layer chromatography (HPTLC)

HPTLC is a major instrumental innovation in the field of separation science and thin layer chromatography is sensitive and robust both qualitatively and quantitatively. It consists of a semi-automatic applicator Linomat IV equipped with 100 µl syringe (Camag, Switzerland). The sample was applied in the form of bands of 8 mm width on aluminium plates precoated with silica gel $60F_{254}$ (20 x 10 cm with 0.25 mm thickness, Merck, Germany) used as stationary phase. The plates were developed in a twin trough glass chambers (Camag, Switzerland) saturated with the mobile phase chloroform : ethanol (9:1 v/v) and a scanner3 with optional deuterium, tungsten and mercury emitting a continuous spectrum of 190 to 800 nm as a source for reflectance scanning densitometry and evaluation of the compound at its λ_{max} 298 nm.

Micro preparative thin layer chromatography

Sample solution of 1 mg/ml concentration was applied as a single band of 170 mm width with a constant flow rate of 6 µl/s on 60 F_{254} silica gel plates. The twin trough chambers were saturated with the mobile phase, chloroform: ethanol in the ratio of 9:1 (v/v) and linear ascending development was carried up to 70 cm from the application level. The developed plate was visualized in a UV cabinet for the detection of the separated compounds. The desired compounds appearing as broad bands at different R_f values were scrapped carefully. The obtained compounds were eluted in micro columns with same mobile phase, chloroform: ethanol (9:1 v/v) to isolate the desired compound from silica gel. The collected fractions were concentrated by evaporating the solvent in a rotavapor at a temperature of 35 to 40°C and analyzed by H^1 NMR and MASS spectroscopy to distinguish and characterize the separated compounds.

RESULTS AND DISCUSSION

Scope of the work and method optimization

As a first step, two sets of media (MS and B5) have been optimized, relating to growth of biomass and yield of secondary metabolites. Both media were found to be potentially active and facilitated the calculation of CIF. The extent of callus proliferation was determined visually and the results are depicted in Figure 3. The CIF was more in both MS (70%) and B5 (63%) media supplemented with NAA (9.0 µM) and BAP (0.9 µM). With IBA (9.0 µM) and BAP (0.8 µM), lower CIF (22.6%) was observed in MS medium but no callus formation was noticed in B5 medium. The callus obtained was profuse, creamy yellow, friable and fast growing, although it was friable but compact with other combinations of growth regulators. As evident from the data in Table 1, highest cell dry weight of 1.02 mg

Table 1. Effect of growth regulators on biomass of *C. congesta* bulb explants cultured on MS and B5 media.

Growth regulators concentration (µM)	MS		B5	
	FW (mg)	DW (mg)	FW (mg)	DW (mg)
2,4-D + BAP (9 + 0.9)	3.23 ± 0.201	0.31 ± 0.032[b]	2.10 ± 0.211	0.15±0.011[d]
2,4-D + BAP (4.5 + 0.6)	4.56 ± 0.334	0.42 ± 0.054	2.30 ± 0.256	0.17 ± 0.015
2,4-D + Kinetin (9 + 0.8)	-	-	-	-
2,4-D + Kinetin (9 + 0.5)	-	-	-	-
NAA + BAP (4.5 + 0.46)	5.45 ± 0.420	0.72 ± 0.071	2.11 ± 0.378	0.31 ± 0.025
NAA + BAP (9 + 0.9)	6.78± .511[a]	1.02 ± 0.138	3.53 ± 0.510[c]	0.40 ± 0.028
NAA + Kinetin (8 + 0.75)	3.66 ± 0.222	0.45 ± 0.066	2.00 ± 0.257	0.35 ± 0.030
IBA + BAP (8 + 0.75)	4.13 ± 0.318	0.56 ± 0.072	-	-
2,4D + NAA + BAP (4.5 + 6.5 + 0.65)	4.67 ± 0.287	0.50 ± 0.051	3.00± .612[c]	0.39 ± 0.033
2,4D + NAA + BAP (6.5 + 4.5 + 0.65)	6.23±0.509[a]	0.73 ± 0.091[b]	4.32 ± 0.615	0.51±0.045[d]
2,4D + NAA + Kinetin (2 + 1 + 0.25)	-	-	-	-
2,4D + BAP + Kinetin (8 + 0.5 + 0.5)	3.97 ± 0.330	0.41 ± 0.074	2.45 ± 0.292	0.51 ± 0.038

FW, Fresh weight; DW, dry weight. Numbers with the same letters in each column means differences are not significant (P > 0.05).

and secondary metabolite content was obtained in MS media supplemented with NAA (9.0 µM) and BAP (0.9 µM), followed by 2, 4-D, NAA and BAP (6.5 + 4.5 + 0.65 µM) whereas, the maximum dry weight of 0.51 mg was obtained in B5 medium supplemented with 2, 4-D, NAA and BAP (6.5 + 4.5 + 0.65 µM). Further increase in concentration of growth regulators did not in any way further enhance a quantifiable growth in biomass formation. In contrast, 2,4-D and kinetin combination resulted in no callus formation proving that NAA and BAP combination is crucial and effective in the induction of callus and enhanced production of bioactive compounds which is in agreement with those of published reports (Zhao et al., 2001; Dhar and Joshi, 2005; Liu et al., 2006).

Plants are rich source of bioactive constituents which include alkaloids, glycosides, flavonoids and volatile oils with wide range of phytochemical activity, and most of the secondary metabolites which are responsible for this activity can be alternatively produced by plant cell cultures for their enhanced production (Ramachandra and Ravishankar, 2002; Vanisree et al., 2004). It is also well known that the specified composition of nutrient medium with mutual ratio of auxin to cytokinin plays a major role not only in successful establishment of cell cultures but also in the production of secondary metabolites (Narayanaswamy, 1994). Therefore, it is necessary to find an optimal combination of various media and they have to be frequently tested for each and every new system. The present study facilitated an attractive offer in this direction and successful results were derived.

Extraction yield and time

Bulb explants cultured showed appreciable callus induction at the end of 40 to 45 days of culture. The dried callus was extracted and analyzed by HPTLC. The peaks obtained at R_f 0.12, 0.32 and 0.56 for standards and the same obtained for callus extract confirms the separated compound as SA, SC and PA respectively (Figure 4), which appeared as very bright fluorescent spots. The compounds isolated by micropreparative TLC were subjected to NMR and Mass spectroscopic studies and when compared with those of standard reference samples and that of published reports, it confirmed that the compounds under investigation were SA, SC and PA (Kopp et al., 1996). It was found that SA and PA content in the crude extract were found to be 1.34 and 3.94 mg/g DW whereas, in callus extract, it was 2.35 and 4.51 mg/g DW (Table 2) which indicates that tissue culture studies in our first attempt facilitated a reasonable enhancement in the formation of the main and most important secondary metabolite, SA and PA. The major toxic elements, SC and DAS that actively contribute to the highly toxic nature of *C. congesta* were 2.08 and 7.69 mg/g DW, respectively, which were found to be slightly enhanced in callus extracts, that is, 3.3 and 8.62 mg/g DW, respectively.

Proscillaridin A

^{13}CNMR, 400 MHz (ppm): 162.6, 149.3, 147.8, 147.2, 122.9, 121.4, 115.2, 109.6, 86.5, 77.7, 74.2, 73.7, 72.4, 72.4, 50.0, 48.9, 48.4, 42.9, 40.1, 38.0, 36.0, 33.6, 32.8, 29.2, 29.0, 27.5, 21.5, 19.3, 17.0, 16.8.

Scillaren A

^{13}CNMR, 400 MHz (ppm): 162.6, 149.3, 147.8, 147.2, 122.9, 121.4, 115.2, 110.4, 109.6, 86.6, 86.5, 81.5, 77.7, 76.8, 74.1, 73.8, 72.3, 71.8, 71.5, 62.2, 50.0, 48.9, 48.4, 42.5, 40.1, 38.0, 36.0, 33.6, 32.8, 29.2, 29.0, 27.5, 21.5, 19.3, 17.3, 16.8.

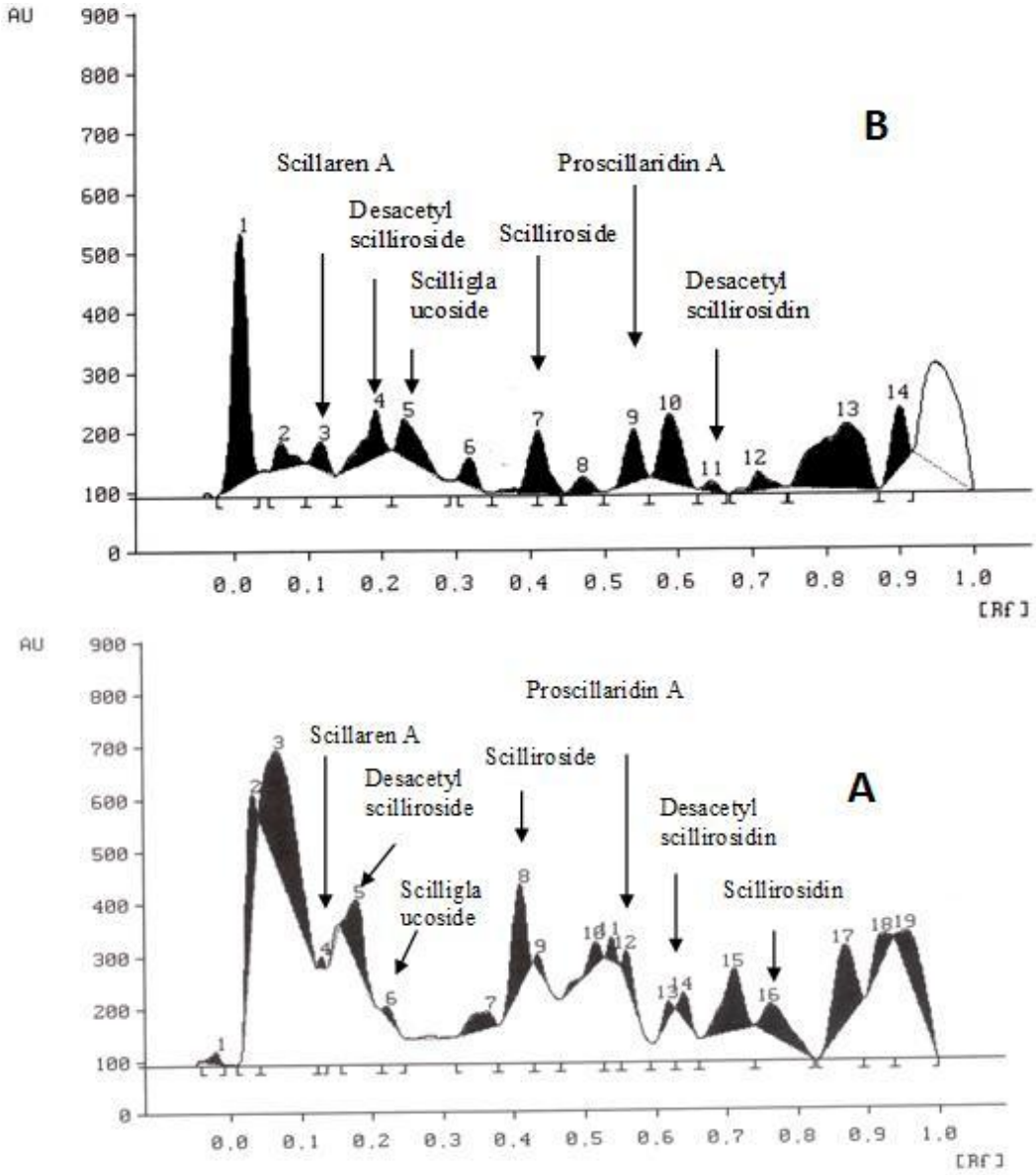

Figure 4. Densitograms showing separation of individual components in (A) Crude extract, (B) Callus extract by reflectance scanning densitometry on HPTLC plates.

Scilliroside

^{13}CNMR, 400 MHz (ppm): 170.2, 162.6, 149.3, 147.0, 142.0, 126.5, 122.9, 115.2, 109.9, 95.1, 81.5, 80.7, 76.8, 74.2, 71.5, 70.5, 62.2, 50.3, 48.5, 47.6, 40.4, 39.6, 36.3, 34.9, 32.1, 29.5, 29.3, 27.5, 21.9, 21.1, 19.6, 17.8, 17.1.

Conclusion

Adopting tissue culture for the enhancement of comercially important and potentially valuable secondary metabolites from a phytochemical source is a rational approach and a very practical strategy for micropropagation studies. The authenticity as has been observed from the information gathered is comprehensive and supports the need of the present study of the Indian herbal based formulation to evaluate allelochemical activity. Evaluation of the results led to the selection of optimal conditions for the induction of callus and production of proscillaridin A, scilliroside and desacetylscilliroside by *in vitro* culture of *C. congesta*. The results obtained are consistent, promising and indicate that the *in vitro* cultures of *C. congesta* might represent an excellent method to produce the bioactive compounds of commercial significance in great yields. Further investigations are underway to scale up the process in a bioreactor to achieve high growth rates and accumulation

Table 2. Composition of Secondary metabolites in crude and callus extracts of *C. congesta* obtained by calculating average peak areas from HPTLC chromatograms

Compound*	R_f	Composition in Crude extract (mg/g DW)	Composition in Callus extract (mg/g DW)
Scillaren A	0.12	1.38	2.35
Desacetylscilliroside	0.18	7.69	8.62
Scilliglaucoside	0.23	1.40	2.68
Scilliroside	0.32	2.08	3.30
Proscillaridin A	0.56	3.94	4.51
Desacetylscillirosidin	0.62	2.16	1.02
Scillirosidin	0.77	3.67	-

*All the compounds were identified using standard reference samples obtained from Merck Chemicals, India. Crude extract was prepared from bulbs using the same procedure like preparation of callus extract

of secondary metabolites.

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