Somatic Embryogenesis of *Hygrophila schulli* (Buch. Ham) M. R. & S.M. Almeida

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Abstract

Hygrophila schulli (Acanthaceae) a tropical herb, is an important medicinal plant, primarily used as diuretic, haematinic, liver tonic, aphrodisiac and anti-cancer agent. Present work deals with the somatic embryogenesis of the plant. Subculturing of well developed compact calli obtained from cotyledonary leaf and cotyledonary node in the regeneration medium of MS supplemented with BA (1-2 mg/l) singly or in combination with NAA (0.1 mg/l), induced the appearance of large number of embryoids in the form of green tubercles all over their surfaces. 1mg/l BA used singly yielded the best result. Embryoids were initially studied under a stereobinocular microscope and their bipolar nature was subsequently confirmed from Scanning Electron Micrographs. Excised embryoids, grown in basal medium, developed into vigorous plantlets. Hardening and acclimatization were done following standard methods.

Keywords : *Hygrophila schulli*, Callus, Regeneration, Embryoid, Somatic-embryo.

INTRODUCTION

Hygrophila schulli (Buch. Ham) M. R. & S. M. Almeida, a tropical herb belonging to the family Acanthaceae, is traditionally used as a folk medicine in India. Extracts of various parts of the plant are used as diuretic, haematinic, liver tonic, aphrodisiac and anti-cancer agent (Chopra *et al*., 1956; Nayar, *et al*., 1989; Kirtikar and Basu, 1935). *H. schulli* is known to contain a number of biologically active compounds such as uronic acid, linoleic acid, lupeol, lupenone, lipids, hygrosterol, polysaccharides, mucilage, minerals and two aliphatic esters (Chopra *et al*., 1958). Efficacy of the plant extract has also been demonstrated in mammalian systems (Santha & Ayer, 1967; Gomes *et al*., 2001; Shailajan *et al*., 2005; Ahmed *et al*., 2001; Mazumdar *et al*., 1997). Due to its significant medicinal importance *H. schulli* is being over-exploited from wild source. Also, loss of habitats due to spread of civilization has become a threat for sustenance of the species. Moreover, seeds in natural condition, while remaining in husk, become nonviable in course of three months (Pal and Arefin, 2007). However, strategies for micropropagation of the plant have not yet been worked out. The present paper reports for the first time a protocol for somatic embryogenesis of *H. schulli* from cotyledonary leaf- and cotyledonary node-derived calli. The technique enables suitable conservation as well as mass propagation of the valuable herb.

MATERIAL AND METHODS

Mature seeds collected from the natural population were allowed to germinate on moist sand or moist filter paper on a petridish in normal temperature (30 ± 2^0C) and diffused natural light in laboratory

condition. About 5 to 10 days old seedlings were used as source of explants. The seedlings were first washed in running tap water for 5 minutes to remove sand from the surface and then rinsed thoroughly in sterile distilled water. Entire seedlings were treated with 0.1% $HgCl_2$ for 2 minutes, then rinsed thoroughly with double- distilled water. Segments of cotyledonary leaf (± 5 mm) and cotyledonary node (± 5 mm) were excised from the young seedlings as explants.

The explants were cultured in MS medium (Murashige and Skoog, 1962) containing 30g/l sucrose and 1% bacteriological agar (Nice). Plant growth regulators (PGRs), 2,4-dichlorophenoxyaceticacid (2,4-D) and N^6-benzyladenine (BA), in different concentrations, singly or in combination, were used for callus induction and growth. However, for regeneration, BA, either singly or in combination with naphthalene acetic acid (NAA), was used. The pH of the medium was adjusted to 5.8 before autoclaving. Borosil glass tubes (22 mm x 150 mm), each containing 20 ml of culture medium and capped with plugs of non-absorbent cotton were autoclaved at 121 °C by 15 psi for 20 minutes. All the cultures were incubated at 25 ± 1^0C and 70 ± 10% RH under 16h photoperiod of about 40 µmol $m^{-2}s^{-1}$ (white fluorescent tube). At least 20 explants for each treatment were tested and the experiments were repeated thrice over a period of three months.

Differentiation was structurally studied initially under a steriobinocular (Wild M3B, Leica) microscope. In course of such studies differentiated structures were excised carefully from the supporting callus tissue by a surgery BP Handle aided with 28 No. blade. For SEM preparation the materials were first rinsed in 0.25 M phosphate buffer (pH 7.2) and fixed in 2.5% glutaraldehyde in same buffer for 2h at 4^0C. Then, after dehydration in a graded series of ethanol and finally with amyl acetate, critical point drying was done in a HCP-2, Hitachi dryer. Finally, the material was coated with gold in an IB_2 ion-sputter-coated chamber (Eco Engineering, Japan) and observed under S-530, Hitachi SEM (Japan). Hardening of regenerated plantlets was achieved following standard methodologies (Smith, 2005).

RESULT AND DISCUSSION

Cotyledonary leaf and cotyledonary nodes were found to be most suitable for callus initiation. For callus induction, 0.2 mg/l 2, 4-D together with 0.2mg/l BA was most effective. Initiation of callus was noticed at about two weeks of culturing. The resultant calli were compact, nodular and greenish white (fig.1, A). The calli continued to grow until the depletion of the medium when those turned brown. 2, 4-D (0.2 mg/l) alone produced little amount of callus followed by multiple root formation in cotyledonary leaf and cotyledonary node explants.

Well-developed compact calli, after four weeks of growth, were transferred to regeneration medium (i.e., basal medium supplemented with various concentrations of BA singly or in combination with NAA). Regeneration was obtained in calli at 4-5 weeks of subculturing in medium containing 1-2 mg/l BA singly or in combination with 0.1 mg/l NAA (table- 1). Greenish tubercles of varied number appeared over the calli (fig.1, B & C). Most effective hormone supplement in this regard was 1.0 mg/l BA; 26 ± 3 tubercles were counted over a callus of ±18mm in diameter. It was also observed that BA together with NAA caused more undifferentiated growth but less tubercle formation. Moreover, when calli were transferred to basal

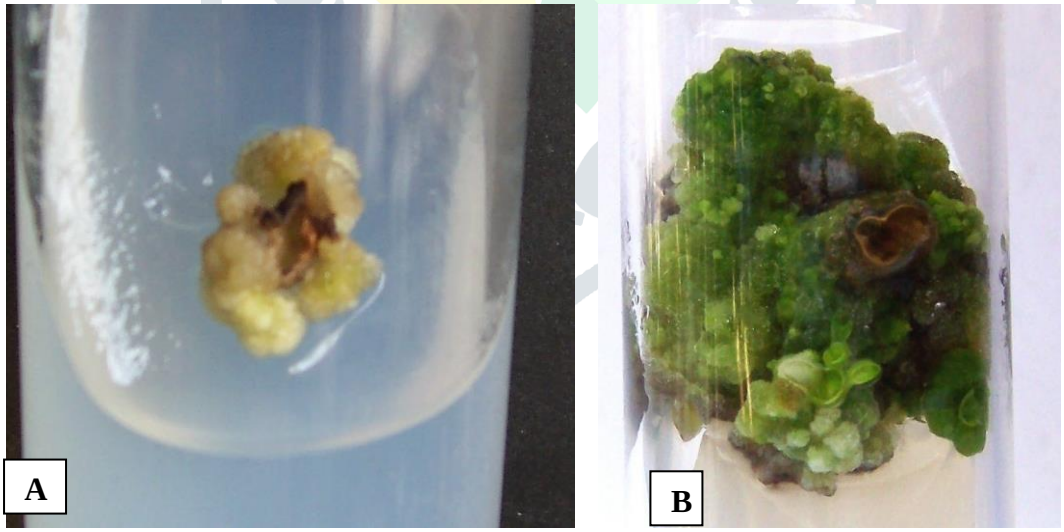
medium without any hormone supplements tubercle formation was obtained though in lesser number than those in medium containing optimum concentration (1mg/l) of BA.

Table- 1: Effect of plant growth regulators as MS supplements on plantlet induction from cotyledonary leaf and cotyledonary node-derived calli of *H. schulli* after 8 weeks of subculturing.

Callus explants	Media combination	% Response	Mean no. of shoots	Mean shoot length (cm)
Cotyledonary leaf	MS	61.10 ± 0.24	5.33 ± 0.47	2.12 ±0.07
	MS + 1 mg/l BA	100 ± 0.00	19.83 ± 0.89	2.70 ± 0.06
	MS + 2 mg/l BA	69.44 ± 0.16	9.50 ± 0.95	1.53 ± 0.05
	MS +0.1 mg/l NAA + 1 mg/l BA	77.78 ±0.47	7.83 ± 0.69	1.70 ± 0.06
	MS +0.1 mg/l NAA + 2 mg/l BA	88.89 ± 0.47	12.86 ± 0.69	2.08 ± 0.07
Cotyledonary node	MS	55.60 ± 0.31	5.8 ± 0.89	2.08 ± 0.10
	MS + 1 mg/l BA	100 ± 0.00	17.50 ± 1.25	2.80 ± 0.06
	MS + 2 mg/l BA	71.10 ± 0.19	9.0 ± 0.81	1.40 ± 0.06
	MS +0.1 mg/l NAA + 1 mg/l BA	77.55 ± 0.31	7.17 ± 1.06	1.57 ± 0.05
	MS +0.1 mg/l NAA + 2 mg/l BA	82.22 ± 0.41	10.33 ±1.21	2.11 ± 0.09

Values are mean ± SD

EXPLANATION OF FIGURE - 1



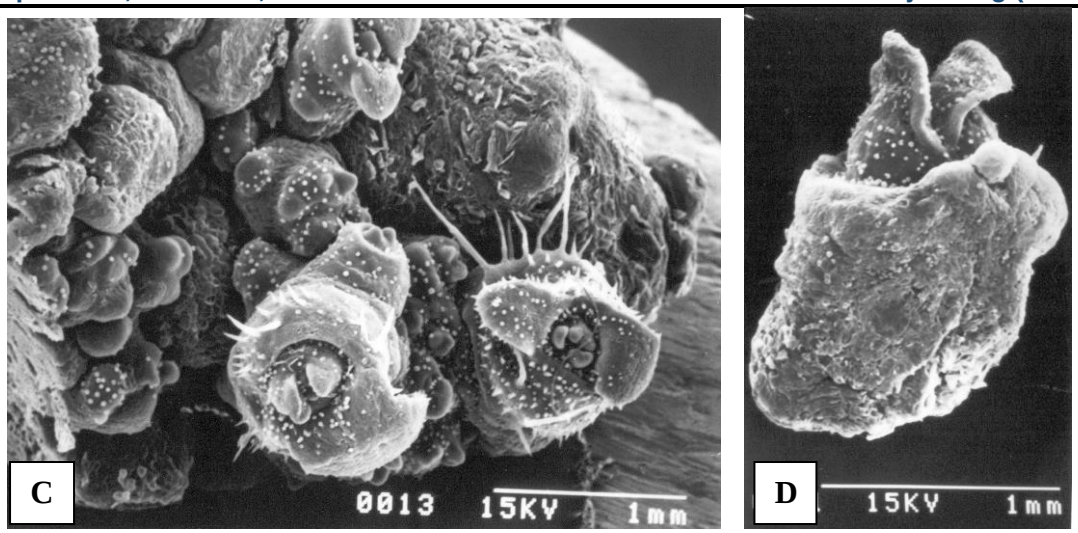


Fig.1, A – D Calli and somatic embryos of *Hygrophila schulli*.
A. Compact nodular callus obtained from cotyledonary leaf after four weeks of culturing, x 1.75. **B.** Initiation of somatic embryos, appearing as green tubercles, over a cotyledonary leaf-derived callus after subculturing for 4 weeks in regeneration medium (MS with 1mg/l BA), x 2. **C.** Scanning Electron Micrograph of callus surface bearing somatic embryos at various stages of development, root and leaf primordia are visible in relatively developed ones depicting their bipolar nature, x 18. **D.** An excised embryo with the pair of cotyledonary leaves, x 40.

Tubercles of various stages of development, carefully excised from the callus tissue, were studied under stereobinocular microscope. At initial stages, those appeared as hemispherical bodies without any apparent tissue differentiation. However, relatively developed tubercles exhibited some sort of differentiation having structures appearing as juvenile shoots and roots. Bipolar nature of the tubercles was confirmed from scanning electron micrographs. Closely set leaf primordia, at various stages of development, were clearly visible at the summit of each tubercle (fig.1, C & D). Roots, sparsely emerged from the base, were stout and devoid of root hairs. Moreover, sparsely distributed unicellular and multicellular trichomes, conical and globular in shapes respectively, were visible all over the surface of the tubercles (fig.1, C). As revealed from SEM studies, bipolarity of the tubercles, having apical leaf primordia and basal root primordia, clearly indicates that the structures were somatic embryos as found earlier in safflower (Mandal and Dutta Gupta, 2002), bermudagrass (Chaudhury and Qu, 2000) and *Dalbergia sissoo* (Das *et al*, 1997).

From the present investigation it can be concluded that cytokinin (BA) promotes somatic embryogenesis in calli of *H. schulli* and auxin (NAA) imparts an inhibitory effect.

The embryoids developed into plantlets, when the calli bearing embryoids were allowed to grow in the regeneration medium or transferred to the basal medium. However, the rate of growth of plantlets was better in the basal medium. Juvenile plantlets were excised from the callus of 6-8 weeks of growth and transferred to the basal medium. Majority (>90%) of those grew with appreciable vigor. By following usual

methods of hardening, the *in vitro* grown plants were found to exhibit survival rate of 86.66% in field condition and did not show any phenotypic variation from those in wild populations. In view of high survival value of the regenerated plants, the procedure could be easily adopted for large-scale cultivation as well as the production of artificial seeds of *Hygrophila schulli*.

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