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RESEARCH ARTICLE

IN VITRO REGENERATION OF *HYGROPHILA SCHULLI* (BUCH. HAM) M. R. & S.M. ALMEIDA THROUGH CALLUS MEDIATED SOMATIC EMBRYOGENESIS

[1*]Pankaj Kumar Pal, [1]Arefin, M. B. and [2]Nirmalya Banerjee

[1]Department of Botany [UGC Centre of Advanced Study], Burdwan University, Burdwan, West Bengal- 713 104, India
[2]Department of Botany, Visva-Bharati, Santiniketan-731 235, India

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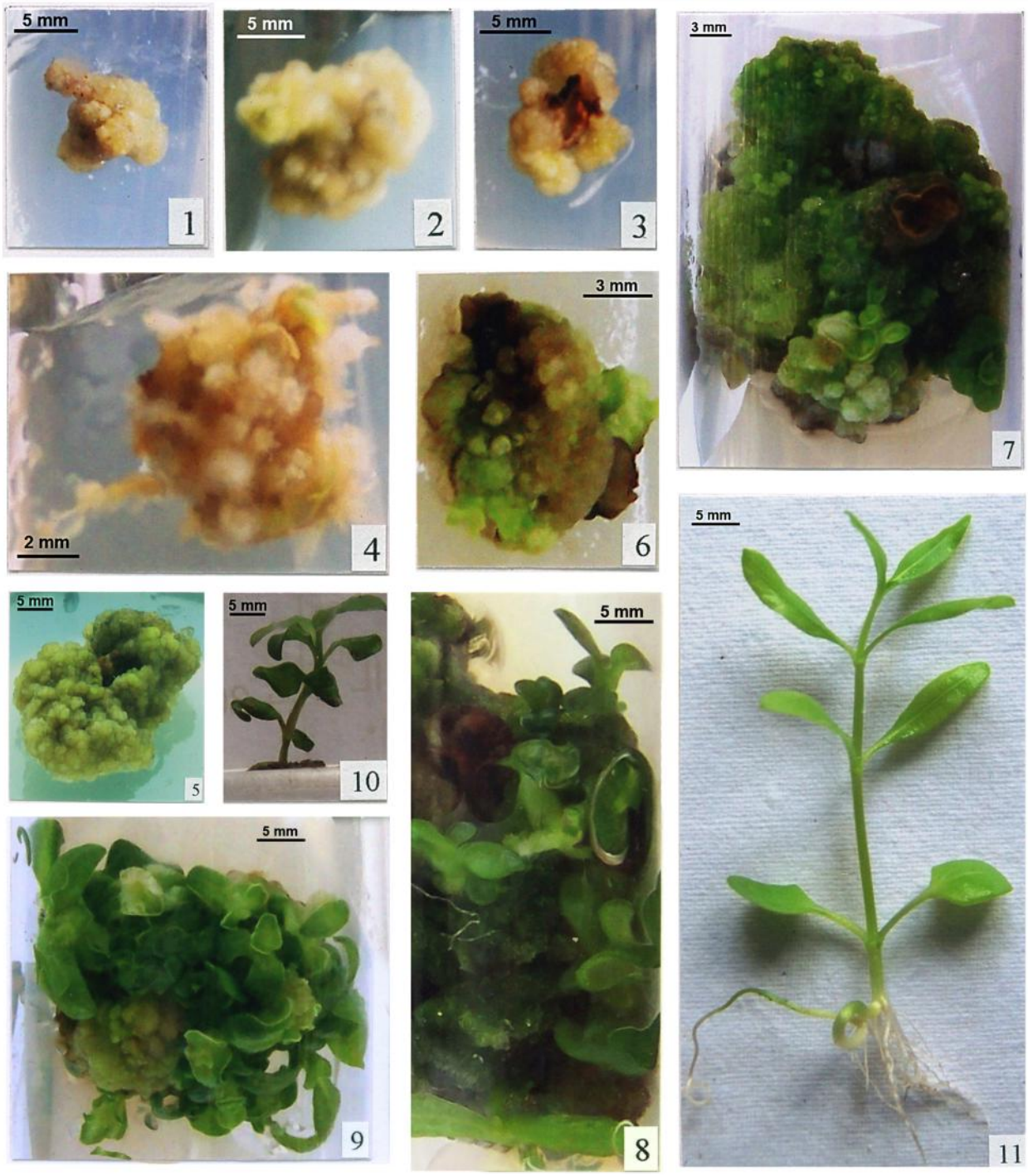
ABSTRACT

Hygrophila schulli, a tropical herb belonging to the family Acanthaceae, is an important medicinal plant, primarily used as diuretic, haematinic and liver tonic. Present work deals with the strategies for the *in vitro* propagation of the species. Well developed compact calli were obtained when cotyledonary leaf and cotyledonary node explants were cultured in MS medium with 0.2 mg/l 2,4-D together with 0.2 mg/l BA. Subculture of the callus in the regeneration medium of MS supplemented with BA (1-2 mg/l) singly or in combination with NAA (0.1 mg/l) induced 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 through scanning electron micrography. Excised embryoids grown in basal medium developed into vigorous plantlets bearing healthy shoots and well ramified roots. Hardening and acclimatization of the regenerants were acomplished following standard methodologies.

INTRODUCTION

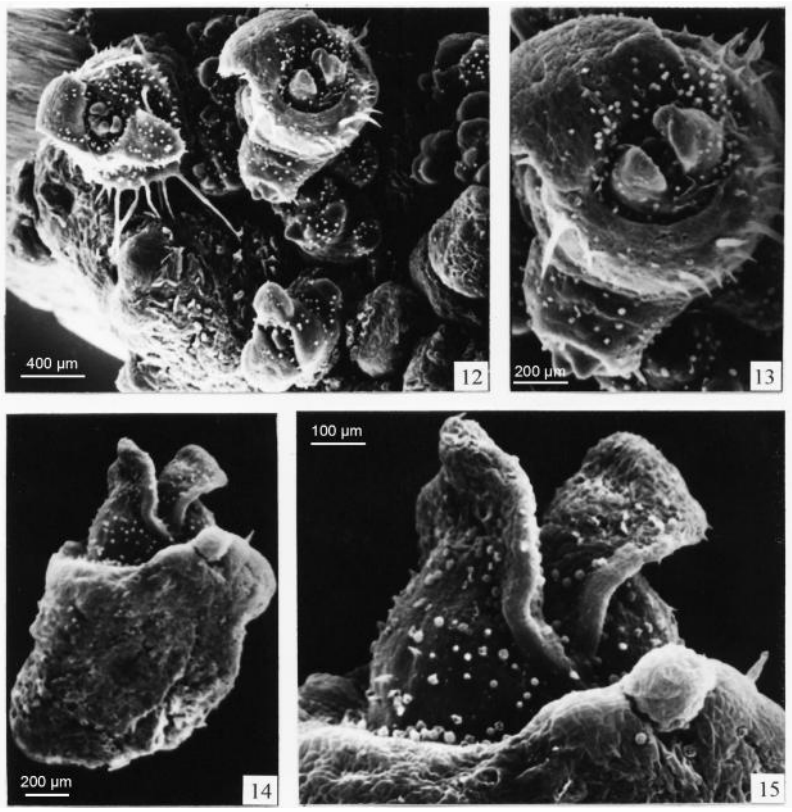
Hygrophila schulli (Buch. Ham) M. R. & S. M. Almeida, belonging to the family Acanthaceae, is traditionally used as a folk medicine in India. Generally the decoctions of various parts of the plant are used. Root extract is diuretic, anti-inflammatory, aphrodisiac and also used in dropsy of chronic Bright's disease, hyperdipsia and vesical calculi. Leaf extract is effective against diarrhoeas, dysenteries, thirst, urinary calculi, urinary discharge, cough, jaundice, urogenital infections, gleet, lumbago and pain in joints. Seeds are used for treatment of anemia, gonorrhoea, spermatorrhoea, sexual inability, weakness, burning sensation and also as abortive (Kirtikar and Basu 1935; Chopra *et al.* 1956; Nayar *et al*., 1989). Plant decoction with paste of long papers (3:2) is used in dropsy and root paste with country liquor (3:1) cures body pain. Seed paste with honey (2:1) can prevent tubercular fistula (Pal and Jain 2000). Crude extract of leaves of *Hygrophila schulli* with flowers of *Butea monosperma* are given with milk for the treatment of leucorrhoea and the juice is given to induce sterility in women (Dhiman 2003). Efficacy of the plant extract as diuretic (Santha and Ayer 1967; Sridharan *et al.* 1981), liver tonic (Shailajan *et al*., 2005), and anti-cancer (Ahmed *et al*. 2001) has been demonstrated in mammalian systems. Also, the petroleum ether extract of root exhibits antitumor activity in Ehrlich ascites carcinoma and Sarcoma-180 bearing mice (Mazumdar *et al.*, 1997). Besides, the plant is known to possess hypoglycemic (Feranando *et al*., 1991), hepatoprotective (Thakur *et al*., 1991; Singh and Handa 1995; Mazumder *et al*. 1996), haematinic (Gomes *et al.*, 2000), anti-inflammatory, antinociceptive (Shanmugasundaram and Venkataraman 2005), antibacterial (Boily and Vanphyvelde 1986) and antifungal (Venkitaraman and Radhakrishnan 1972) activities. *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). Due to its significant medicinal importance, *H. schulli* is being continuously over exploited from wild source in the past. 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. *In-vitro* techniques can enable the formulation of suitable conservation strategies as well as mass propagation of this valuable herb from a minimum of plant material, thereby, facilitating the production of large quantities of biomass available throughout the year. Anthesis, pollen germination, seed dormancy and seed germination of this plant species have been worked out from time to time by several workers (Amritphale *et al*., 1993; 1995; Arefin and Pal 2004). However, work on any aspect of *in-vitro* culture of the planthas not yet been done. The present paper reports for the first time an effective protocol for *in-vitro* plantlet regeneration of *H. schulli* from calluses, derived from different explants, through somatic embryogenesis.

Corresponding author: Pankaj Kumar Pal,
Department of Botany [UGC Centre of Advanced Study], Burdwan University, Burdwan, West Bengal- 713 104, India



EXPLANATION OF PLATE- 1

Fig.1: Hypocotyl-derived friable callus after four weeks of culturing, x1.25.
Fig.2: Cotyledonary node-derived compact, nodular callus after four weeks of culturing, x 1.6.
Fig.3: Compact nodular callus obtained from cotyledonary leaf after four weeks of culturing, the central brown portion is the remnant of explant, x 1.75.
Fig.4: Formation of multiple roots induced by low concentration (0.2 mg/l) of 2, 4-D in a cotyledonary leaf-derived callus, x 3.75.
Fig.5: Callus in figure 3 after 8 weeks of culturing, x 1.75.
Fig.6: Initiation of somatic embryos appearing as green tubercles over a cotyledonary node-derived callus after subculturing for 4 weeks in regeneration medium (MS with 1mg/l BA), x 3.
Fig.7: Somatic embryos initiated over cotyledonary leaf-derived callus after subculturing for 4 weeks in regeneration medium, x 2.2.
Fig.8: Regenerated plantlets over a cotyledonary leaf-derived callus after 4 weeks of transfer of the embryoids bearing stage from the regeneration medium to the basal medium, x 2.5.
Fig.9: Regenerated plantlets over the cotyledonary node-derived callus in figure 6 after 4 weeks of transfer of the embryoids bearing stage from the regeneration medium to the basal medium, x 2.5.
Fig.10: Regenerated plantlets under acclimatization, grown in thermocol cup containing sterile sand and organic manure, x 1.
Fig.11: A relatively developed regenerated plant from research plot, x 1.5.



EXPLANATION OF PLATE- 2

Figs. 12 – 13: SEM micrographs of somatic embryos developed on cotyledonary leaf-derived callus of *H. schulli*.
Fig.12: 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 30.
Fig.13: One of the relatively developed embryo enlarged, sparsely distributed trichomes are visible all over the surface, x 50.
Fig.14: An excised embryo with the pair of cotyledonary leaves, x 40.
Fig.15: Part of the material in figure 14 magnified showing globular and conical trichomes over the surface, x 100.

Table 1. Effect of plant growth regulators as MS supplements on callus induction from cotyledonary leaf, cotyledonary node and hypocotyl explants of *H. schulli* after 8 weeks

Explants	Media combination	% Response*	Types of callus
Cotyledonary leaf	MS + 0.2 mg/l 2, 4-D	66.67±0.58	c, w, lw (Multiple root)
	MS + 0.1 mg/l 2, 4-D + 0.1 mg/l BA	41.11±0.35	f, w, lw
	MS + 0.2 mg/l 2, 4-D + 0.1 mg/l BA	64.44±1.37	c, g, lg
	MS + 0.2 mg/l 2, 4-D + 0.2 mg/l BA	86.66±0.94	c, g, lg
	MS + 0.5 mg/l 2, 4-D + 0.2 mg/l BA	46.66±1.06	c, p, md
	MS + 2 mg/l 2, 4-D + 0.5 mg/l BA	30.00±0.50	c, w, lw
Cotyledonary node	MS + 0.2 mg/l 2, 4-D	73.33±0.58	c, w, lw (Multiple root)
	MS + 0.1 mg/l 2, 4-D + 0.1 mg/l BA	30.00 ±1.08	f, p, lw
	MS + 0.2 mg/l 2, 4-D + 0.1 mg/l BA	57.77 ±1.50	c, g, md
	MS + 0.2 mg/l 2, 4-D + 0.2 mg/l BA	93.33 ±2.21	c, g, lg
	MS + 0.5 mg/l 2, 4-D + 0.2 mg/l BA	30.00 ±0.50	f, p, lw
	MS + 2 mg/l 2, 4-D + 0.5 mg/l BA	No response	-
Hypocotyl	MS + 0.2 mg/l 2, 4-D	No response	-
	MS + 0.1 mg/l 2, 4-D + 0.1 mg/l BA	No response	-
	MS + 0.2 mg/l 2, 4-D + 0.1 mg/l BA	32.50 ±0.70	f, w, lw
	MS + 0.2 mg/l 2, 4-D + 0.2 mg/l BA	45.83 ±0.85	f, w, lw
	MS + 0.5 mg/l 2, 4-D + 0.2 mg/l BA	17.77 ± 1.07	f, w, lw
	MS + 2 mg/l 2, 4-D + 0.5 mg/l BA	No response	-

* Values are mean ± SD
[c- compact, f- friable, w- white, p- pale green, g- greenish white, lg- large, lw- low, md- moderate,]

Table 2. 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 initiating Explants	Media combination	% Response	Mean no. of shoot	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

MATERIALS AND METHODS

Mature seeds of *H. schulli* were collected from the natural population as well as plants grown in the research plot. Freshly collected seeds 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. Seedlings were treated with 0.1% $HgCl_2$ for 2 minutes, then rinsed thoroughly with double- distilled water. Segments of cotyledonary leaf (± 5 mm), cotyledonary node (± 5 mm) and hypocotyl (± 5 mm) were excised from the young seedlings as explants. The explants were cultured in MS semisolid 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 induction and growth of callus. 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 cultures were incubated at 25 ± 1^0C and 70 ± 10% relative humidity under 16h photoperiod of about 40µmol $m^{-2}s^{-1}$ (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 of organs was morphologically 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).

RESULTS AND DISCUSSION

Seeds, sowed under diffused natural light conditions at 30±2^0 C temperature, imbibed water normally and swelled nicely. As the seed coat contains mucilage, naturally it absorbs water and swells immediately after sowing. Seed started to germinate within 1-4 days of sowing. Germination was epigeal. Of the three explants tested i.e., cotyledonary leaf, cotyledonary nodes and hypocotyl segments, the former two were found to be most suitable for callus initiation. Various concentrations of 2, 4-D and BA singly or in combinations were employed for callus induction. Among those, 0.2 mg/l 2, 4-D together with 0.2mg/l BA was found to be most effective for callus initiation and growth in case of both cotyledonary leaf and cotyledonary node explants. Initiation of callus was noticed at about two weeks of culturing. The resultant calli were compact, nodular and greenish white (Figs 2, 3 & 5). The calli continued to grow until the medium is depleted which subsequently 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 (Fig. 4).

Hypocotyl segments showed poor response with most of the hormone combinations tried. In this case, the combinations of 2, 4-D and BA in different concentrations produced very low amount of friable white callus (Fig. 1). The observations reveal that for successful callogenesis of *Hygrophila schulli*, auxin alone is not sufficient. Cytokinin (BA) together with auxin is required, as found earlier in *Albizia lebbeck* (Das *et al*., 1995), Bermuda grass (Chaudhury and Qu 2000), *Andrographis alata* (Nagaraja *et al.,* 2003). However, in those plants, the ratio of optimum concentrations of auxin and cytokinin was quite high (≥ 2) in contrast to *H. schulli* where the ratio was merely one. Relative efficiencies of different explants for callogenesis in response to various hormone supplements are summerised in Table-1. Callus developed from all the three types of explants, 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). None of the hormone supplements tried imparted differentiation in hypocotyl-derived callus. By 8 weeks such calli turned brown and dried up. On the other hand regeneration was obtained in callus derived from cotyledonary leaves and cotyledonary nodes 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- 2). Greenish tubercles of varied number appeared over the callus mass (Figs 6, 7). 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 callus were transferred to basal medium without any hormone supplements, tubercle formation was obtained though lesser in number than those in medium containing optimum concentration (1mg/l) of BA.

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 apex of each tubercle. 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 (Figs 12-15). As revealed from SEM studies, bipolarity of the tubercles, having apical leaf primordia and basal root primodia, clearly indicated that the structures were somatic embryos as found earlier in safflower (Mandal and Dutta Gupta 2002), bermuda grass (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. When the calli bearing embryoids were allowed to grow in the regeneration medium or transferred to the basal medium, the embryoids developed into plantlets.

However, the rate of growth of plantlets was better in the basal medium (Figs 8, 9). In both the cases, only a few plantlets tend to mature in comparison to their large number over a callus. It happens perhaps due to their competition in a relatively small space available over the callus. In view of that, juvenile plantlets were excised from the callus of 6-8 weeks of growth in the regeneration medium and were transferred to the basal medium. Majority (>90%) of those grew with appreciable vigour. As the ultimate success of *in-vitro* propagation lies in the establishment of plants in soil under natural condition, the regenerated plantlets were transferred to thermocol cups containing sterile sand and organic manure. Those were initially kept in temperature (25 ± 1^0C), relative humidity ($70 \pm 10\%$) and photoperiod (under 16h, about 40 µmol $m^{-2}s^{-1}$) controlled room for about two weeks. Shoot elongation associated with growth of leaves took place (Fig. 10). Subsequently the cups were kept in the net-house for about ten days. Then the plantlets were transferred to earthen pots containing autoclaved field soil and after a week finally planted in the research field (Fig. 11). The *in vitro* grown plants exhibited survival rate of 86.66% in field condition and did not show any phenotypic variation from those in wild population.

Conclusion

Cotyledonary node and cotyledonary leaf segments, as explants of *Hygrophila schulli* are quite suitable for induction of compact nodular callus. Auxin (0.2mg/l 2,4-D) together with cytokinin (0.2mg/l BA) is most effective PGR supplement for callogenesis. Cytokinin (1mg/l BA) induces somatic embryogenesis. However, for further growth of the embryoids and their development to plantlets simply the MS basal medium alone is sufficient; no hormone supplement was required. In course of acclimatization 86.66% success can be achieved for regenerated plantlets. In view of high survival value of the regenerated plants, the procedure could be easily adopted for *ex situ* conservation and mass-scale propagation of the invaluable medicinally important plant, *Hygrophila schulli*.

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