CHAPTER-7

IN VITRO REGENERATION OF *Streptocaulon sylvestre* WIGHT

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7.1 INTRODUCTION

In vitro regeneration is the most commercially successful area of plant biotechnology and has introduced an exciting new phase into plant propagation and breeding. The potential for regenerating a whole plant from a single cell was first hypothesized by the German botanist Haberlandt in 1902. However, it was not until 1950's that whole plants were regenerated from isolated cultured cells. In the past 40 years or so since Haberlandt's principle of totipotency of single plant cell was realized, the field of plant tissue, cell and organ culture has grown to be of immense size, importance and scope (Thorpe, 1978; Vasil *et al.*, 1979; Hicks, 1980; Wilkins & Dodds, 1983).

Development of micropropagation techniques in various laboratories have demonstrated that with the help of these techniques huge number of plantlets could be produced in a shorter time compared to the other conventional methods of propagation (Pierik, 1993). It is particulary more suitable for rare and endangered plant species. During the last few years tissue culture techniques have been extensively exploited not only for obtaining virus free healthy plantlets, but also for rapid and large scale propagation of endangered taxa which is otherwise hard to propagate by conventional method of propagation.

One of the major advantages of a perfect tissue culture system involving the culture of either callus or a cell suspension is that, in a model system, one can culture a small piece of callus or volume of cell suspension to produce any desired quantity of cells, then change the culture conditions to induce either somatic embryogenesis or adventitious shoot formation and obtain complete plants.

The success in the technology and application of *in vitro* methods is largely due to a better understanding of the nutritional requirements of cultured cells and tissues (Murashige, 1974; Gamborg *et al.* 1976; Street, 1977; Phillips & Collins, 1979; Evans *et al.* 1981) which varies from species to species.

Successful induction of callus formation using various explants like stem, pith, cambium, root, leaf, cotyledon, hypocotyle, epicotyle etc. has already been reported which makes it clear that all living nucleated plant tissue can give rise to a proliferating callus when excised and placed on a suitable culture medium. However, much difficulty is often experienced in establishing a callus from previously

unattempted, differentiated source of tissue, for there is apparently considerable diversity in nutritional requirement of tissue from different species, or even from different explants within same plant.

Development of efficient protocols for initiation of germination and growth of seedlings is a must for successful application of tissue culture techniques in any fundamental and applied problems of plant sciences. (Datta *et al*, 1999).

With refinement in tissue culture techniques various methods have been developed by different groups using different explants and different media for successful regeneration of plant species (Goh, 1989; Sagawa, 1989; Arditti & Ernst, 1993). Following these techniques, the commercial growers have produced countless 'clones' (Champagnat *et al.* 1966, 68; Biveins & Hacket, 1969; Thompson, 1971; Ueda & Torikata 1972). However, the extensive literature giving detailed information of various plant tissues cultured under *in vitro* conditions contain absolutely no data on the medium composition and culture conditions necessary for growth and regeneration of *Streptocaulon sylvestre* Wight.

Streptocaulon sylvestre Wigth, a perennial prostrate branching herb of Asclepiadaceae, is very rare and endangered plant of India. Only a few plants are now found growing in two fields with thick grass cover within the campus of North Bengal University. It now appears that the species is endemic to the Terai region of Darjeeling in Eastern India. The strictly prostrate habit of the plant with slightly woody but easily breakable stem, which never produces any adventitious root from nodes and/or internodes but always grows with grasses.

S. sylvestre is propagated by seeds. The major constraints of this conventional propagation method are the very low reproductive capacity and high rate of seedling mortality in natural habitat. Moreover, owing to increasing exploitation of the natural population by biotic factors and since, plant grows as wild only and not cultivated, it is facing the threat of extinction. It now seems that the natural mode of multiplication is far from adequate to save this endemic and highly endangered plant. Therefore, large scale mutiplication through tissue culture remains the only alternative to save this plant. Till date, reports on *in vitro* seed germination and seedling growth is entirely lacking.

The present investigation was undertaken to find out the nutritional requirements and suitable culture conditon for *in vitro* seed germination and /or rapid mass propagation in *S. sylvestre* with an ultimate objective of conserration of this taxon. The Materials and methods used for this work has been mentioned in Chapter-2.

7.2 RESULTS AND DISCUSSION

7.2.1 *IN VITRO* SEED GERMINATION

Induction of seed germination of *Streptocaulon sylvestre* under *in vitro* condition was observed in MS medium at different pH levels. (Table-7.1 & Plate-VI).

Effect of pH of different levels showed that seed germination could be induced from all levels of pH in MS medium. However, the degree of response and time requirement for germination varied. The best response (90.00 %) was obtained from pH 5.4 medium. High percentage of seed-germination (85.33 %) was also recorded in pH 5.6, while pH 3.6 yielded lowest (37.04 %) percentage of seed germination. The time requirement for completing the germination in different pH levels ranged from 12.40 to 14.80 days. However, germination was first noticed after 5 th day of sowing at pH 5.4 and 5.8 in MS medium when the radicle came out by rupturing the seed coat. Initially, the radicle was white in colour but turned creamish within 5-6 days following germination.

Table-7.1 ***In vitro*** **seed germination of** ***S. sylvestre*** **at different pH levels**

pH	Time required for complete the germination ± SE	Percentage of seed germination ± S
3.6	14.80 ± 0.73	37.04 ± 3.71
4.0	14.60 ± 1.03	65.66 ± 2.77
5.4	12.60 ± 1.43	90.00 ± 4.08
5.6	14.00 ± 1.30	85.33 ± 3.74
5.8	12.40 ± 0.93	67.95 ± 2.53
6.2	13.60 ± 1.07	58.66 ± 3.74
7.0	13.20 ± 0.73	50.00 ± 3.16

7.2.2 CALLUS INDUCTION

Performance of callus induction by various explants like epicotyle, hypocolyle and colyledon was studied in details and the observations are presnted in Table-7.2 & Plate-VI. Cotyledon was found to be most suitable explant for callus induction in *Streptocaulon sylvestre* as highest percentage of callusing was recorded when they were inoculated in the nutrient medium. Although callusing was also noticed in epicotyle and hypo cotyledonous explants but was not satisfactory (Table-7.2). Time requirement for callus

Table-7.2 Effect of growth regulators on callus induction from different explants of *Streptocaulon sylvestre.*

Hormon	Concen-tration mg / l	Cotyledon		Hypocotyle		Epicotyle	
		% of explant Survived ± SE	% of explant produced calluse ± SE	% of explant Survived ± SE	% of explant produced calluse ± SE	% of explant Survived ± SE	% of explant produced calluse ± SE
IAA	0.2	52.50 ± 2.50	50.00 ± 4.08	24.66 ± 2.44	21.33 ± 1.61	47.33 ± 5.20	47.33 ± 5.20
	0.5	35.00 ± 2.88	30.00 ± 4.08	33.71 ± 1.82	33.71 ± 1.82	39.33 ± 3.05	34.33 ± 2.77
	1.0	27.50 ± 2.50	25.00 ± 2.88	40.00 ± 3.16	40.00 ± 3.16	33.33 ± 4.56	28.33 ± 2.04
	2.0	35.00 ± 2.88	32.50 ± 2.50	34.33 ± 2.77	26.33 ± 2.99	32.66 ± 3.36	32.66 ± 3.36
	3.0	45.00 ± 2.88	45.00 ± 2.88	35.00 ± 4.08	29.99 ± 2.04	46.00 ± 2.45	46.00 ± 2.45
2, 4 - D	0.2	77.00 ± 1.22	77.00 ± 1.22	77.28 ± 1.75	73.28 ± 3.69	74.61 ± 2.56	74.61 ± 2.56
	0.5	86.40 ± 1.75	86.40 ± 1.75	81.64 ± 2.24	81.64 ± 2.24	80.50 ± 2.00	80.50 ± 2.00
	1.0	80.81 ± 1.81	80.81 ± 1.81	78.00 ± 1.22	78.00 ± 1.22	74.33 ± 2.15	74.33 ± 2.15
	2.0	74.00 ± 3.67	74.00 ± 3.67	70.57 ± 2.76	70.57 ± 2.76	67.95 ± 2.53	67.95 ± 2.53
	3.0	73.61 ± 2.21	69.61 ± 2.85	69.66 ± 3.51	65.66 ± 2.77	66.00 ± 3.67	61.00 ± 4.00
NAA	0.2	23.71 ± 2.23	20.85 ± 1.99	25.00 ± 3.87	21.00 ± 1.00	20.38 ± 2.18	17.52 ± 1.10
	0.5	30.38 ± 2.85	30.38 ± 2.85	30.00 ± 4.18	30.00 ± 4.18	32.04 ± 2.53	32.04 ± 2.53
	1.0	26.00 ± 3.67	22.00 ± 1.22	26.00 ± 3.67	26.00 ± 3.67	26.71 ± 3.48	26.71 ± 3.48
	2.0	23.66 ± 2.60	20.33 ± 1.33	26.00 ± 3.67	22.00 ± 6.44	19.66 ± 1.53	19.66 ± 1.53
	3.0	27.00 ± 3.39	18.00 ± 4.63	19.85 ± 1.69	19.85 ± 1.69	24.00 ± 1.00	19.00 ± 4.85

formation was lowest (5.8 days) in colytedonous explants while hypocotyle and epicolyle takes 7-12 days and 15-25 days, respectively, for callusing.

The first visible change in all the explants was swelling at the cut end which is followed by the formation of undifferentiated green mass of cells- the callus. The colour of callus varies from creamy white to light green. Callus produced from colyledon was creamy white in colour. The callus was soft and fragile and healthy. To study the effect of hormones on callus formation different concentrations of various auxins like Indole acetic acid (IAA; 0.2, 0.5, 1.0, 2.0 &3.0 mgl^{-1}) *a* napthalene acetic acid (NAA; 0.2, 0.5, 1.0, 2.0 & 3.0 mgl^{-1}) and 2,4- dichlorophenoxy acetic acid (2,4-D; 0.2, 0.5, 1.0, 2.0 & 3.0 mgl^{-1}) was added to MS medium. Highest percentage of callusing was obtained when the colyledons were cultured on MS medium supplemented with 0.5 mgl^{-1} 2,4-D. 86.40,

81.64 and 80.50 percent callus formation was noted from cotyledon, hypocotyle and epicolyle, respectively, when inoculated in the said combination (MS + 0.5 mgl^{-1} 2,4-D). IAA and NAA induces callusing at 0.2 mgl^{-1} and 0.5 mgl^{-1} concentrations, respectively. However, the percentage of callus formation was low in comparison to 2,4-D. The callusing percentage at different concentrations of IAA & NAA is shown in Table-7.2.

Incorporation of IAA to MS medium induced nodular callus on the upper cut ends of the hypocotyle explants. These nodular structures directly produces shoots. IAA induced multiple shoots formation was also observed at the proximal embedded end in cotyledon and directly in the exil of the epicotyle explants.

The frequency of direct regeneration without forming callus varied with the dose of IAA and the type of explant. The highest frequency of direct shoot regeneration was observed from hypocotyle explants followed by epicotyle and cotyledons. IAA at 0.5mg/l concentration was found to be optimal for obtaining maximum frequency of direct shoot formation from hypocotyle explants, IAA at 1.0 mg/l concentration showed moderate response in shoot regeneration from hypocotyle explants. However, the maximum number of shoots were obtained from cotyledon and epicotyle explants in 0.5 mg/l and 0.2 mg/l IAA concentration, respectively. IAA also showed normal rooting and the best result was observed in IAA of 1.00 mg/l from hypocotyle explant.

Skoog and Miller (1957) first pointed out that growth regulator concentrations in the culture medium are critical for the control of growth and morphogenesis. However, many physical factor have also been recognized as equally important (Murashige, 1977, 1984, 1990). In the present study growth regulators were found to be essential for both callus induction and regeneration. Among the auxins, 2,4-D was effective for callus induction in all three tested explants. 2,4-D was indispensable for induction and growth of callus from tissues of Asclepiadaceae has been reported earlier in *Tylophora indica* (Bera & Roy, 1996). In the present material 2,4-D at 0.5 mg/l was optimum for callus induction from cotyledon, hypocotyle and epicotyle explants.

The fact that different concentrations of 2,4-D are necessary for callusing from root, scutellum, cotyledonary nodes, coleoptile and leaf sheath nodes has also been confirmed in rice (Wu & Li 1971). For callus induction 2,4-D was found to be suitable in a number of pulses e.g. *Glycine*; Beverdorf & Bingham, 1977, *Phaseolus vulgaris* (Lieu & Boll, 1970; Mok & Mok, 1977), *Medicago sativa* (Saunders & Bingham, 1972), *Melilotus alba* (Taira *et al.* 1977), *Trifolium hybridum* (Schenk & Hildebrandt, 1972) *Trifolium subterraneum* (Graham, 1968) *Lathyrus sativus, Cicer arietinum, Dolichos biflorus* (Sinha *et al.* 1983).

EXPLANATIONS OF PHOTOGRAPHS

Plate VI.

***Seed Germination, Callusing and regeneration of plantlets of Streptocaulon sylvestre* under *in vitro* condition.**

Photo 1. Young seedling obtained through *in vitro* seed germination.

Photo 2. Profuse callusing from epicotyle in 0.5 mgl^{-1} 2,4-D supplemented MS medium.

Photo 3. Profuse callusing from epicotyle in 0.5 mgl^{-1} 2,4-D supplemented MS medium.

Photo 4. Profuse callusing from epicotyle in 0.5 mgl^{-1} 2,4-D supplemented MS medium.

Photo 5. Callus obtained from hypocotyle.

Photo 6. Direct regeneration of plantlelts from hypocotyledonous explants.

Photo 7. Direct regeneration of plantlelts from hypocotyledonous explants

Photo 8. *In vitro* rooting.

Photo 9. Young seedling obtained through *in vitro* seed germination.

PLATE VI


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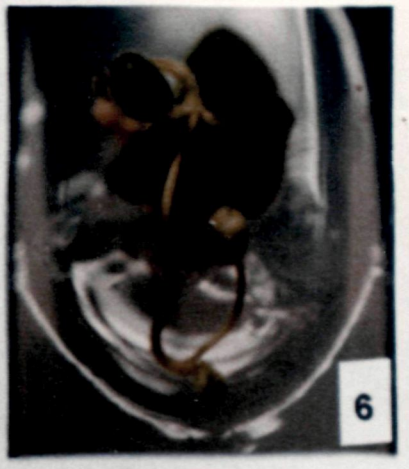
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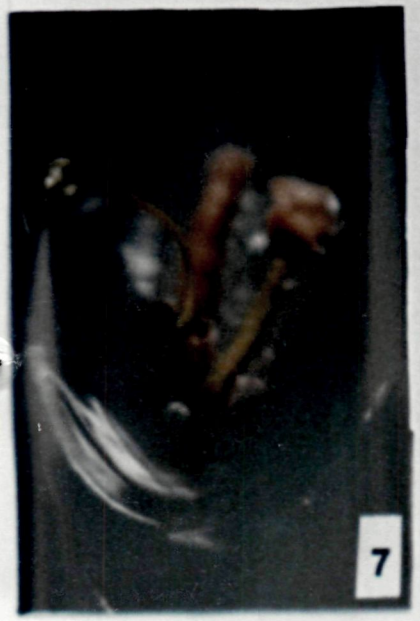
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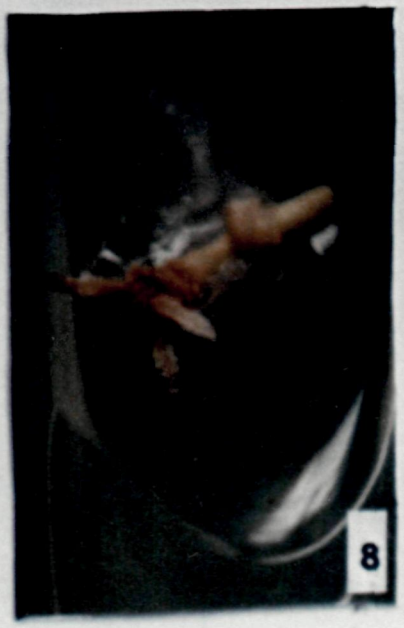
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Shoot buds were mostly formed from the cut edges of the hypocotyles but more profusely from the end which was in direct contact with the medium. This may be due to presence of cambial tissue in this region. Direct organogenesis from explants was previously reported from leaf explants of *Tylophora indica* (Asclepiadaceae). (Bera & Roy, 1993, 1996). *Rauwolfia serpentina* (Mitra & Chaturvedi, 1970), *Anthurium* sp. (Pierik & Steegmans, 1976) and from hypocotyle explants of *Peganum harmala* (Saini & Jaiwal, 2000).

In terms of rooting efficiency IAA at a concentration of 1.00 mg/l was found to be most effective. For *in vitro* rooting IAA was found to be suitable in a number of species. eg. *Tylophora indica* (Sharma & Chandel, 1992; Bera & Roy, 1993, 1996). *Cordyline terminalis* and *Dracaena godseffianae* (Miller & Murashige, 1976), *Pelargonium* sp. (Cassels *et al.* 1980). *Santalum album* (Lakshmi Sita *et al.* 1979).

The present study is the first and only attempt to find out the nutritional and hormonal requirement for callusing and regeneration from different explants of *S. sylvestre*. The results obtained in this study clearly indicate the fact that the *in vitro* mass propagation system could be very effective for this endangered plants. Conservation through micropropagation has now become very popular to the scientists and successful application of this in *S. sylvestre* can save this plant from being extinct.