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In vitro propagation through root-derived callus culture of *Swertia chirata* Buch.-Ham. ex Wall

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A procedure for regeneration of complete plantlets of *Swertia chirata* via indirect organogenesis is described. Callus was obtained from *in vitro* regenerated roots on Murashige and Skoog (1962) (MS) medium supplemented with varying concentrations of 6, benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D). 13.32 µM BAP in combination with 0.90 µM 2,4-D proved to be the most effective concentration for callus induction, multiplication and adventitious shoot regeneration from callus surface. The optimal hormone combination for shoot multiplication was shown to be 8.88 µM BAP, 2.85 µM indole-3 acetic acid (IAA) and 271.45 µM adenine sulphate (Ads) giving an average of 10.70 shoots after 4 weeks and 17.50 shoots after 8 weeks. Individual elongated shoots were rooted on half-strength MS medium supplemented with varying concentrations of auxins. Best rooting was obtained with 4.90 µM indole-3 butyric acid (IBA) where an average of 14.40 and 21.50 roots per shoot could be obtained after 4 and 8 weeks, respectively. *In vitro* raised plantlets with well developed shoots and roots were acclimatized successfully.

Key words: *Swertia chirata*, *in-vitro* propagation, callus, adventitious shoots, rosette clumps.

INTRODUCTION

Swertia chirata (family Gentianaceae; commonly known as chirata) is an indigenous species of temperate Himalaya, found at an altitude of 1200 to 3000 m from Kashmir to Nepal and also distributed in Bhutan, Khasi hills and Sikkim (The Wealth of India, 1976; Garg, 1987; Kirtikar and Basu, 1998; Pradhan and Badola, 2010). The species is priced for its bitter bioactive compounds viz. amarogentin, xanthones, iridoid glycosides, mangiferin and C-glucoflavones (Dalal and Shah, 1956; Asthana et al., 1991; Jensen and Schripsema, 2002; Mallikarjun et al., 2010). *S. chirata* can be traced through the medicinal history as a safe ethnomedicinal herb. It is used in the treatment of various ailments like chronic fever, malaria,

liver and stomach disorders, cold and cough, asthma and joint pains. It's blood-purifying, antifungal and antihelmintic qualities have also been extensively exploited.

Propagation of *S. chirata* through seeds is hampered by poor seed germination rate (only 2 to 4%), low seed viability and long gestation period (Anonymous, 1997; Rai et al., 2000; Joshi and Dhawan, 2005). This, in addition to indiscriminate extraction of the plants from the wilderness, subsequent habitat losses and lack of adequate commercial plantations have accelerated the genetic erosion of the species leading to it being characterized as critically endangered (Nayar and Shastry, 1990; Semwal et al., 2007; IUCN, 2008; Pradhan and Badola, 2010). Conventional approaches of propagation alone cannot guarantee the re-establishment and recovery of this important plant species. Consequently, the application of alternative reproducible micropropagation strategies has become inevitable for mass propagation and sustainable utilization of this age-old medicinal plant.

There are only some reports on *in vitro* propagation of *S. chirata.* Ahuja et al. (2003), Chaudhuri et al. (2007),

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Koul et al. (2009) and Pant et al. (2010) have reported micropropagation of *S. chirata* via field-grown nodal explants, while seedling derived shoot tip explants were used in the study by Joshi and Dhawan (2007a) and Balaraju et al. (2009). Joshi and Dhawan (2007b) also performed ISSR marker analysis of genetic diversity among *S. chirata* genotypes of temperate Himalayan region of India. Direct shoot organogenesis from *in vitro* leaves and regeneration via immature seed cultures of *S. chirata* was reported by Chaudhuri et al. (2008, 2009). Wang et al. (2009) developed a method for *in vitro* shoot regeneration from leaves taken from field-grown plants. *In vitro* propagation of *S. chirata* from roots has been reported by Wawrosch et al. (1999) and Pant et al. (2010) who described direct shoot regeneration via root explants.

To our knowledge, the present study would be the first report on *in vitro* propagation and mass multiplication of *S. chirata* via root segment derived callus culture.

MATERIALS AND METHODS

Explant source

In our earlier study on *S.chirata* (Pant et al., 2010), shoots were regenerated *in vitro* from nodal explants, further multiplied and subsequently rooted on half-strength MS medium supplemented with 4.90 µM IBA. *In vitro* grown roots harvested from these axenic cultures were used as explant material in the present study. Eight weeks old roots were aseptically removed from *in vitro* regenerated shoots and repeatedly washed in sterile distilled water to remove agar adhering to the surface. proximal, middle and distal root segments were excised from these roots and used as explants for callus induction.

Induction and development of callus

Various combinations of phytohormones viz. BAP and 2,4-D were tested for their effect on callus formation on root explant. For this purpose, 10 mm sections of roots were incised and placed horizontally on agar gelled (0.7% w/v) MS medium and supplemented with 3% sucrose and different concentrations of BAP (4.44 to 22.20 µM) in combination with 2,4-D (0.90 to 4.52 µM). MS medium lacking growth regulators served as control. After 5 weeks of incubation, observations were recorded. Data on callus were scored as the percent of the root explant forming callus and degree of callusing. Response percent of *in vitro* root induced callus formation was calculated as:

$$\text{Response (\%)} = \frac{\text{Number of explants in which callus was induced}}{\text{Total number of explants cultured}} \times 100$$

Degree of callusing = + (poor callusing); ++ (moderate callusing); +++ (good callusing); ++++ (very good callusing).

Multiplication and differentiation of callus (shoot organogenesis)

The callus obtained was transferred onto full-strength MS medium fortified with combinations of BAP (4.44 to 13.32 µM) and 2,4-D

(0.90 to 2.26 µM). Data on organogenic response percent and multiplication rate of callus was scored after 4 weeks, while observations on number of shoots induced per callus clump and length of differentiated shoots were recorded after 5 weeks of incubation. Organogenic calli were transferred on to maintenance medium. Two parameters were used to assess organogenic response of callus as:

$$\text{Organic response of callus (\%)} = \frac{\text{Number of organogenic cultures}}{\text{Total number of explants cultured}} \times 100$$

$$\text{Multiplication rate} = \frac{\text{Final fresh weight of callus}}{\text{Initial weight of callus inoculated}} \times 100$$

In vitro shoot multiplication

Regenerated shoots were used to assess the effect of growth regulators on further *in vitro* shoot multiplication. Full strength MS medium supplemented with varying concentrations of BAP (4.44 to 13.32 µM) alone and in combination with IAA (0.57 to 2.85 µM) and/or adjuvant Ads (271.45 µM) was tested for further multiplication of adventitious buds differentiated from root induced callus. Single shoots of size which were up to 1 cm were used for this purpose. Observations pertaining to average shoot number and average shoot length were recorded after periodic intervals of 4 and 8 weeks. Routine sub-culturing of multiple shoots was carried out at periodic interval of every 4-weeks.

In vitro rooting

Experiments on *in vitro* rooting of *in vitro* raised shoots were attempted with 2 to 3 cm long shoots developed during shoot multiplication. These experiments were initiated after 180 days of sub-culturing to get maximum material for testing different hormone concentrations. Shoots were cultured on MS medium containing 2% sucrose and supplemented with auxins IAA (1.14 to 11.40 µM), IBA (0.98 to 9.80 µM) and NAA (1.07 to 10.74 µM). Single shoot was cultured for initiation of roots and observations pertaining to mean number of roots produced and mean root length were recorded after 4 and 8 weeks interval.

Medium and culture conditions

The pH of medium was adjusted to 5.8 (using 1.0 N HCl or 1.0 N NaOH) before adding 0.7% agar (w/v) and sterilized by autoclaving at 15 lbs (1.8 kg/cm^2) pressure at 121°C for 15 min. Cultures were incubated in culture room at 25 ± 1°C temperature and 60 to 65% relative humidity. To assess the culture conditions required for induction and multiplication of callus, explants were incubated both in complete dark conditions (16 h) and under a 16/8 h (light/dark) photoperiod with light supplied by cool-white fluorescent tubes (Philips, India) at an intensity of 2500 lux. All other experiments pertaining to *in vitro* shoot differentiation, shoot multiplication and *in vitro* rooting were carried out under a photoperiod of 16 h light and 8 h dark, maintained using photoperiodic stimulators.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to 1/4 strength MS medium having 2% sucrose devoid of PGR for 7 days in flasks. Thereafter, they were transferred to polybags containing a

mixture of soil : sand : manure (1:1:1), covered with perforated polythene bags and kept under agronet-shade house conditions. Acclimatized plants were later shifted to polybags filled with soil.

Statistical analysis

All experiments were repeated thrice. Each treatment consisted of minimum of ten replicates. The data was analysed using analysis of variance (ANOVA) of completely randomized design (CRD) in GenStat 5 Edition 3.2 for PC/Windows NT {Copyright 1995, Lawes Agricultural Trust (Rothamsted Experimental Station)}. The significance level was determined at 5% ($p \leq 0.05$), 1% ($p \leq 0.01$) and 0.1% ($p \leq 0.001$). Mean values of treatments were compared with least significance difference (LSD).

RESULTS

Initiation and development of callus

Observations after 2 to 3 weeks of incubation revealed enlargement of most of the root explants. Little incisions made on the explants resulted in the formation of callus. Callus induction occurred on all media combinations (Figure 1A). Final observations after 5 weeks showed that MS medium + 13.32 µM BAP + 0.90 µM 2,4-D proved to be most efficient in inducing caulogenic response in maximum (70%) explants with maximum degree of callusing (Figure 1B).

Callus growth and differentiation (shoot organogenesis)

Callus obtained was further multiplied on MS medium fortified with different concentrations of BAP and 2,4-D (Table 1). All the treatments tried were significantly superior to control as no callus multiplication occurred in medium devoid of any plant growth regulator. The multiplied callus exhibited shoot organogenesis in 4 to 5 weeks after 2 to 3 sub-cultures. Shoots developed through organogenesis were excised and used for further *in vitro* shoot multiplication. This organogenic callus was sub-cultured again for callus multiplication and shoot formation after every 4 weeks. For sub-culturing initially, 50 mg of callus was inoculated for callus multiplication and shoot regeneration. Best callus multiplication rate of 11.63 folds was obtained in treatment 4 (13.32 µM BAP + 0.90 µM 2,4-D). Optimal amount of yellowish-white callus was obtained on this medium. The same media combination also gave the best organogenic response with maximum number of 8.10 shoots produced per callus clump in 100% of the callus cultured (Figure 1B).

Furthermore, all cultures were maintained on MS medium supplemented with 13.32 µM BAP and 0.90 µM 2,4-D and regular sub-culturing was carried out every 4 weeks to multiply the callus and to maintain the organogenic potential of callus. Sub-culture interval of more than 5 weeks resulted in the formation of brownish non-organogenic callus with a general decline in callus growth rate (data not shown). Organogenic callus multiplied and produced new shoots only under light conditions. Callus sub-cultured on organogenic medium and kept under dark conditions did not multiply and no shoot differentiation from callus was recorded.

In vitro shoot multiplication

Adventitious shoots regenerated from callus were in the form of rosette clumps and needed elongation (Figure 1C). For this, a pulse treatment of GA_3 (1.16 µM) was given and elongation was observed within 14 days. Subsequently, elongated shoots were used for *in vitro* multiplication (Table 2). Results in the present study showed the necessity of plant growth regulators for *in vitro* shoot multiplication as the shoots cultured on basal MS medium (devoid of any PGR) did not multiply and died after some time.

Shoot number and length (as observed after 4 and 8 weeks) varied significantly in all the treatments tested. On BAP supplemented medium, results were significantly superior to the data recorded on medium containing Kn. Treatment 3 (8.88 µM BAP) gave an average of 6.30 shoots (1.02 cm shoot length) after 4 weeks and 9.30 shoots (1.58 cm shoot length) after 8 weeks. Comparatively, on medium fortified with Kn alone, a maximum of only 4.80 shoots (mean shoot length 0.99 cm) after 4 weeks and 7.70 shoots (mean shoot length 1.42 cm) after 8 weeks could be obtained in treatment 6 (9.29 µM Kn). Only BAP supplemented medium was used for further experiments on the effect of interactions of auxin IAA and adjuvant Ads.

IAA at 2.85 µM concentration proved to be more effective in enhancing shoot multiplication rate and treatment 12 (8.88 µM BAP + 2.85 µM IAA) gave the best results of all the BAP + IAA combinations tested. Incorporation of adenine sulphate in BAP and IAA supplemented medium resulted in significant improvement in *in vitro* shoot multiplication. Best results with mean number of 10.70 shoots and mean shoot length of 1.89 cm after 4 weeks interval and mean number of 17.50 shoots with mean shoot length of 2.75 cm after 8 weeks period were obtained in treatment 15 (8.88 µM BAP + 2.85 µM IAA + 271.45 µM Ads) (Figure 1D). The results far exceeded the number of shoots and shoot length recorded in all other treatments.

MS medium supplemented with 4.44 µM BAP was used for routine sub culturing of multiple shoots at an interval of every 4-weeks.

In vitro rooting

Response to rooting was different in different concentrations of hormones. All the treatments tested in the present study varied significantly. In treatment 1 (half
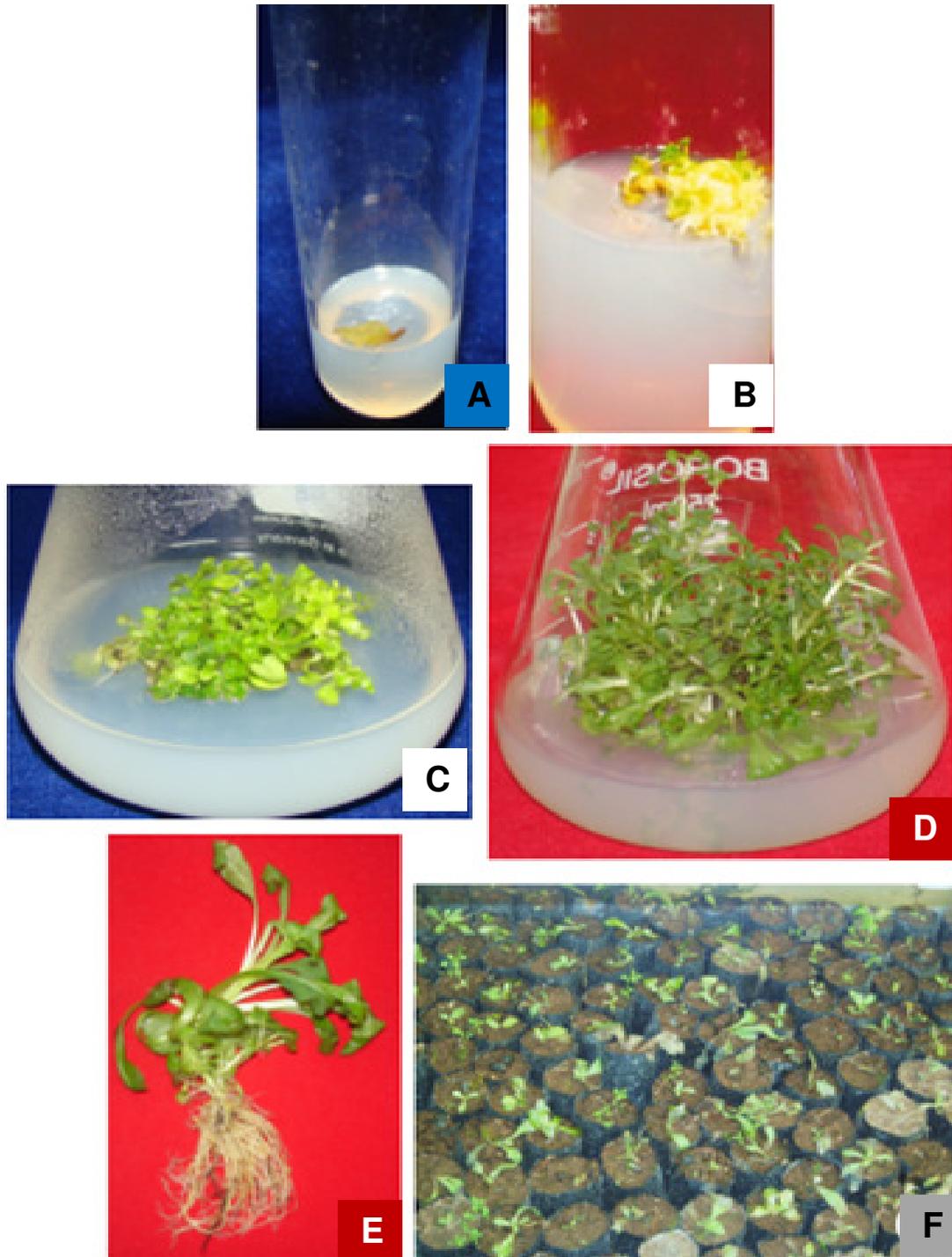
Figure 1. A) Callus induction; B) callus multiplication and shoot organogenesis; C) rosetting of adventitious shoots; D) *in vitro* shoot multiplication on MS + 8.88 µM BAP + 2.85 µM IAA + 271.45 µM Ads; E) *in vitro* regenerated plantlet; F) *Ex vitro* hardening.

strength MS medium without any plant growth regulator), no rooting could be obtained while all other treatments proved effective in root induction and in most of the treatments, root initiation was observed within 10 to 15 days of culture (Table 3).

Maximum rooting was recorded in treatment 12 (4.90 µM IBA) (Figure 1E). The treatment proved to be most efficient for all other treatments tested for *in vitro* rooting of adventitious shoots giving a maximum of 14.40 and 21.50 average number of roots (after 4 and 8 weeks

Table 1. Effect of plant growth regulators' interactions on callus multiplication and shoot organogenesis in *S. chirata*. Data were recorded after 5 weeks (50 mg callus cultured initially).

| Treatment | MS medium + PGR concentration (µM) | | Observation after 4 week | | | Observation after 5 week | | |
	BAP	2,4-D	Fresh weight of callus (mg)	Nature of callus	Callus multiplication rate (fold)	Organogenic response of callus (%)	Mean number of shoot per callus clump	Mean shoot length (cm)
1 (Control)	0.00	0.00	000	-	0.00	0	0.00	0.00
2	4.44	0.90	204	Yellow-white friable	4.08	40	1.50	0.13
3	8.88	0.90	468	Yellow-white friable	9.36	60	2.30	0.32
4	13.32	0.90	581	Yellow-white friable	11.63	100	8.10	0.72
5	17.76	0.90	525	Yellow-white friable	10.49	70	4.70	0.35
6	22.20	0.90	209	Yellow-brown friable	4.18	40	1.50	0.12
7	4.44	2.26	377	Yellow-brown friable	7.53	20	0.80	0.08
8	8.88	2.26	341	Brown-compact	6.82	30	0.80	0.12
9	13.32	2.26	357	Brown-compact	7.13	40	1.60	0.23
10	17.76	2.26	283	Brown-compact	5.66	30	0.80	0.31
11	22.20	2.26	203	Brown-compact	4.06	20	0.40	0.14
Grand mean			323		6.45		2.05	0.23
Significance			***		***		***	***
LSD			155.20		3.10		1.76	0.25

*** - Significance at 0.1%

period) with mean root length of 1.15 and 1.88 cm, respectively. Further increase in the concentration of IBA (9.80 µM) resulted in a decline in mean number of roots per shoot which is 9.60 after 4 weeks, 19.20 after 8 weeks and mean root length of 0.44 cm after 4 weeks and 1.35 cm after 8 weeks.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to liquid 1/4 MS strength medium having 2% sucrose and devoid of any plant growth regulator, for 7 days in flasks. Plantlets with well-developed shoots and roots were subsequently transferred to polybags containing a rooting mixture of soil : sand : manure (1:1:1). These were covered with perforated polythene bags in shade and maintained in agronet shade house for the next 30 days where they exhibited enhanced growth. Hardened plantlets were subsequently transferred to soil in polybags for further growth and development and later exposed to field conditions where a survival rate of over 85% was achieved (Figure 1F).

DISCUSSION

In the present study, callus was induced on *in vitro* root segments using MS medium supplemented with 4.44 to 22.20 µM BAP and 0.90 to 4.52 µM 2,4-D. The combination of a cytokinin with an auxin has been reported to strongly enhance callus induction in dicots (Reinert and Bajaj, 1990; George, 2008). An auxin is generally required for the induction of callus from explants. The auxin most commonly employed to initiate callus cultures is 2,4 -D. The efficiency of 2,4-D in callus induction from root explants has also been reported in *Dacus carota* (Syono, 1965), *Cymbidium ensifolium* (Chang and Chang, 1998), *Ceropegia sahyadrica* (Nikam and Savant, 2007) and *Clitoria ternatea* (Shazad et al., 2007).

In our study, combination of BAP and 2,4-D gave the best results of shoot organogenesis via callus. The efficiency of BAP in shoot induction via callus has been reported to be due to the ability of plant tissue to metabolize natural hormones more

Table 2. Effect of plant growth regulators' interactions on *in vitro* multiplication of adventitious shoots regenerated via root-derived callus of *S. chirata.*

Treatment	MS medium + PGR concentration (µM)				Mean shoot number		Mean shoot length (cm)	
	BAP	Kn	IAA	Ads	After 4 week	After 8 week	After 4 week	After 8 week
1(Control)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	4.44	0.00	0.00	0.00	4.40	5.50	0.98	1.27
3	8.88	0.00	0.00	0.00	6.30	9.30	1.02	1.58
4	13.32	0.00	0.00	0.00	4.70	6.70	1.06	1.45
5	0.00	4.65	0.00	0.00	3.80	6.00	0.79	1.12
6	0.00	9.29	0.00	0.00	4.80	7.70	0.87	1.39
7	0.00	13.95	0.00	0.00	4.50	7.60	0.99	1.42
8	4.44	0.00	1.14	0.00	4.40	5.10	1.09	1.32
9	8.88	0.00	1.14	0.00	5.10	6.80	1.11	1.70
10	13.32	0.00	1.14	0.00	5.30	6.90	1.21	1.47
11	4.44	0.00	2.85	0.00	4.70	6.50	1.61	2.11
12	8.88	0.00	2.85	0.00	6.20	7.70	1.68	2.16
13	13.32	0.00	2.85	0.00	6.10	7.40	1.55	1.79
14	4.44	0.00	2.85	271.45	7.80	16.50	1.79	2.42
15	8.88	0.00	2.85	271.45	10.70	17.50	1.89	2.75
16	13.32	0.00	2.85	271.45	6.50	10.50	1.34	1.91
Grand mean					5.33	7.98	1.19	1.62
Significance					***	***	***	***
LSD					1.18	1.83	0.33	0.43

***Significance at 0.1%.

Table 3. Effect of different auxins and their concentrations on rooting of *in vitro* regenerated adventitious shoots of *S. chirata* developed via indirect organogenesis.

Treatment	1/2 strength MS medium +Auxin (µM)			Average number of root per shoot		Average length of root (cm)	
	NAA	IAA	IBA	After 4 week	After 8 week	After 4 week	After 8 week
1.(Control)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2.	1.07	0.00	0.00	2.70	6.80	0.22	0.60
3.	2.69	0.00	0.00	3.80	6.50	0.55	0.79
4.	5.37	0.00	0.00	3.70	7.00	0.45	0.85
5.	10.74	0.00	0.00	2.50	4.80	0.38	0.86
6.	0.00	1.14	0.00	3.00	9.20	0.62	0.88
7.	0.00	2.85	0.00	4.40	7.40	0.68	1.05
8.	0.00	5.70	0.00	4.70	9.60	0.81	1.28
9.	0.00	11.40	0.00	3.70	9.40	0.57	1.09
10.	0.00	0.00	0.98	3.40	5.70	0.48	0.85
11.	0.00	0.00	2.46	5.10	8.20	0.68	0.98
12.	0.00	0.00	4.90	14.40	21.50	1.15	1.88
13.	0.00	0.00	9.80	9.60	19.20	0.44	1.35
Grand Mean				4.69	8.87	0.54	0.96
Significance				***	***	***	***
LSD				2.16	3.07	0.24	0.27

***Significance at 0.1%.

readily than artificial growth regulators or due to the ability of BAP to induce production of natural hormones such as zeatin within the tissue and thus work through natural hormone system (Sharma and Wakhlu, 2003). However, the regenerated adventitious shoots were in the form of rosette clumps (Figure 1D). GA_3 is reported to be required for adventitious shoot development from callus once the meristemoids are formed (Jarret and Hasegawa, 1981). Therefore, a pulse treatment of GA_3 for 1 week was given to the shoots and it was found to promote shoot elongation within 14 days. This observation is in con-firmation with previous reports which indicated that GA_3 is conducive for in vitro differentiation of shoot buds and promotes shoot elongation and regeneration in works on Saussurea lappa (Arora and Bhojwani, 1989), Ficus Acacia sinuata (Vengadesan et al., 2000, 2003), Ricinus communis (Ganesh Kumari et al., 2008).

The in vitro regenerated shoots were further multiplied on full-strength MS medium containing 3% sucrose and supplemented with different concentrations and combinations of plant growth regulators. Of the different treatments tested, full strength MS medium fortified with BAP (8.88 µM), IAA (2.85 µM) and Ads (271.45 µM) gave the best results of in vitro multiplication of shoots regenerated from root-derived callus. The positive role of adenine sulphate in multiplication of adventitious shoots has previously been highlighted in some species viz. Psoralea coryfolia (Saxena et al., 1997), Centella asiatica (Patra et al., 1998), Melia azedarach (Vila et al., 2005), Pentamena indicum (Sivanesan and Jeong, 2007), Musa acuminate (Kacar et al., 2010).

In an earlier study on in vitro propagation of S. chirata via root segments procured from in vitro raised seedlings, Wawrosch et al. (1999) reported callusing on cytokinin (2 iP/ BAP/ Kn/ Z) supplemented MS medium. However, they optimized a procedure for shoot regeneration without any intervening callus phase by culturing of root explants on BAP (3 µM) supplemented medium and subsequent transfer on to basal MS medium (for 3 weeks). By this method, they could obtain an average of only 1.9 shoots per explant and the regenerated shoots were vitrified. Low frequency of shoot regeneration, small size and hyperhydration of shoots militates against the use of their protocol. In our study, a mean number of 8.10 healthy shoots per root explants after 4 weeks could be obtained which were further multiplied with 17.50 fold shoot multiplication in 8 weeks.

In the present study, IBA at 4.90 µM concentration proved to be most efficient for rhizogenesis which was regenerated, resulting in long and healthy root system. Documented literature shows that IBA has been found to be superior for in vitro rooting of adventitious shoots in some other species such as Flacourtia jangomas (Chandra and Bhanja, 2002), Exacum travancoricum (Kannan et al., 2007), Clitoria ternatea (Shahzad et al., 2007), Lins culinaris (Omran et al., 2008), Sarcostemma

brevistigma (Thomas and Shankar, 2009), S. chirata (Koul et al., 2009) and Podophyllum hexandrum (Chakraborty et al., 2010). Contrastingly, in some species, basal MS medium devoid of any plant growth regulator proved to be the best for in vitro rooting of root-derived shoots viz. Averrhoa carambola (Kantharajah et al., 1992), Gentiana sp. (Hosokawa et al., 1996), Pothomorphe umbellate (Pereira et al., 2000), Blackstonia perfoliata (Bijelovic et al., 2004). However, in the present investigation, medium lacking any plant growth regulator proved to be completely incompetent for root induction. Wawrosch et al. (1999), in their study on S. chirata via root explants, reported callus formation on transfer of in vitro shoots to rooting medium supplemented with auxin. In contrast, in our study, callusing was not observed in any of the treatments tested and long, healthy roots developed on IBA augmented medium.

Plants growing under tissue culture conditions are semi-autotrophic and susceptible to transplantation shock due to delicate root system, reduced amount of epicuticular wax and reduced or abnormal stomata (Hazarika, 2003). Therefore, the plants need to be properly acclimatized when they are transferred from in vitro conditions to soil. In the present study, in vitro hardening and acclimatization was carried out for two months before transferring them to field conditions. The eight weeks old tissue culture raised plantlets which were directly transferred to polybags containing sand : soil : FYM without hardening and acclimatization showed 0 to 5% survival rate. In contrast, over 85% plantlet survival rate was obtained when plantlets were well-acclimatized prior to transfer to pots containing soil in field conditions. In vitro hardening of ex-agar rooted plantlets was tested in culture room. Regenerated plantlets were first hardened under controlled conditions of culture room for a period of one week on 1/4 X liquid MS medium devoid of any plant growth regulator and sucrose supply was decreased to 2%. Plantlets were placed on absorbent cotton soaked in this liquid medium for supporting root systems. After a week, these plantlets were transferred to a rooting mixture of soil : sand : manure (1:1:1) and covered with perforated polythene bags in net house. Holes were made in polythene bags for gaseous exchange. For acclimatizing the plantlets, bags were withdrawn periodically and on emergence of new leaves, the poly-bags were completely removed. The plants maintained in net house were irrigated with tap-water. During the process, the shoots elongated, leaves turned greener and expanded. Well developed plants were subsequently shifted to polybags filled with soil at High Altitude Herbal Garden, Chakrata (Forest Research Institute) and exposed to field conditions with a good survival percentage.

The present study, thus, connotes first report on in vitro production of S. chirata plants via in vitro raised roots through an intermediary callus phase. The method described is simple, reproducible, affordable and has

been successfully used for recurrent shoot production without loss of multiplication potential in more than two years of 4-week sub-culture. Thus, this package of technology has the potential of increasing the productivity by mass scale propagation of *S. chirata*. High shoot multiplication rate with healthy rooting are attractive features of this study and credited by the following aspects:

1. Root explants being derived aseptically do not face the problem of contamination and are therefore ideal for germplasm exchange and cryopreservation.
2. The system will be useful for biochemical and physiological studies in relation to organ differentiation and genetic improvement studies via indirect organogenesis.

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