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Influence of phytohormones on adventitious shoot regeneration from leaf explants of an endangered Himalayan medicinal plant *Swertia chirayita* Buch.-Hams. ex Wall

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ABSTRACT

A valuable and regenerative protocol of micropropagation with high efficiency was developed through direct organogenesis from leaf explants under *in vitro* conditions. Murashige and Skoog (MS) medium supplemented with various growth hormones combinations resulted in maximum84.26% of shoot bud induction and 88.91% of multiplication. *Swertia* plants are very difficult to hardened with maximum percent survival upto acclimatization and field transfer. Keeping in view for better root induction microshoots were dipped in 1.0 mg/l IBA for 24 hours and then transferred to half strength MS basal medium supplemented with 0.02% activated charcoal resulted 75.21% rooting with roots having a very good growth. Rooted plantlets were hardened gradually in different potting mixtures and then were successfully acclimatized under *ex vitro* conditions.

Key words *: Swertiachirayita, Direct organogenesis, Proliferation, Hardening, Percent rooting, Acclimatization, Medicinal.*

Introduction

Swertia chirayita a highly valuable medicinal plant growswith an altitude range of 1200-3000 m amsl. The plant can be grown in a variety of soils with sandy loam rich in carbon, humus and found in open ground, recently slash and burnt forests (Edwards, 1993). There seen no consistency in the literature where the habit of *Swertia chirayita* being cited. Some authors have described *Swertia chirayita* as an annual (Anonymous, 1982; Kirtikar and Basu, 1984) and others as biennial or pluriannual herb (Edwards, 1993).The entire plant is used in traditional medicines for blood pressure, dyspesia, epilepsy, blood purification, liver disorders,gastrointestinal infection, used to treat malaria and diabetes. The species is in high demandwhich leads to its reckless collection and it causes the decrease in the population of the species to a very low level (Kumari *et al*., 2019). An alternate to limitations of vegetative propagation methods, a novel, easy and fast technique of micropropagation applied for production of a large number of plants(Pant *et al*., 2012; Sharma *et al*., 2016; Kumari

and Kanwar, 2020). But it was observed propagation through indirect organogenesis may leads to some variations. So the aim of the present study was to produce true to type plants through direct organogenesis using immature leaf explants to retain the pharmaceutical value of the *in vitro* raised plants. Even in the literature there are only few reports of direct shoot organogenesis from leaf explants in *Swertiachirayita* Buch.-Hams. ex Wall (Chaudhuri *et al*., 2008; Kumar *et al*., 2018).

Materials and Methods

Adventitious shoot regeneration, proliferation and rooting

Immature leaves were procured from the young and healthy plants of *Swertiachirayita* from nursery of Forest Product Department of Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) (Figure 1). Leaves were cut into small pieces and cultured on solid MS medium supplemented with different concentrations of BA in combination with Kinetin and NAA. The cultures were maintained under 16 hours photoperiod at 25±2 ºC. The shoots obtained were transferred to solid MS medium supplemented with different concentrations of BA, Kinetin, NAA and GA_3 for *in vitro* proliferation and multiplication. *In vitro* raised microshoots of 4.00 to 4.05 cm length were isolated then dipped for 24 hours in different concentrations 1.0-3.0 mg/l IBA after that cultured on half strength MS supplemented with different concentrations of activated charcoal ranging from 0.02% to 0.04%.

Hardening and acclimatization

In vitro raised microshoots of length 3.5 cm to 4.0 cm along with four to six leaves were taken out carefully andwashed with luke warm water. Thereafter, roots of the *in vitro* raised shoots were dipped in 0.2% bavistin solution for 15 minutes to avoid any fungal attack and then washed thoroughly with running tap water and were transferred to plastic cups. The plants were covered with polythene bags to maintain the relative humidity (90-100%). The plants were watered at every alternate day and observed for growth and development. Percent survival of the transferred plants was recorded after every four weeks. Statistical differences between mean tabulated value was estimated using Duncan's multiple range test (DMRT) with the SPSS Software for window version 16.0.

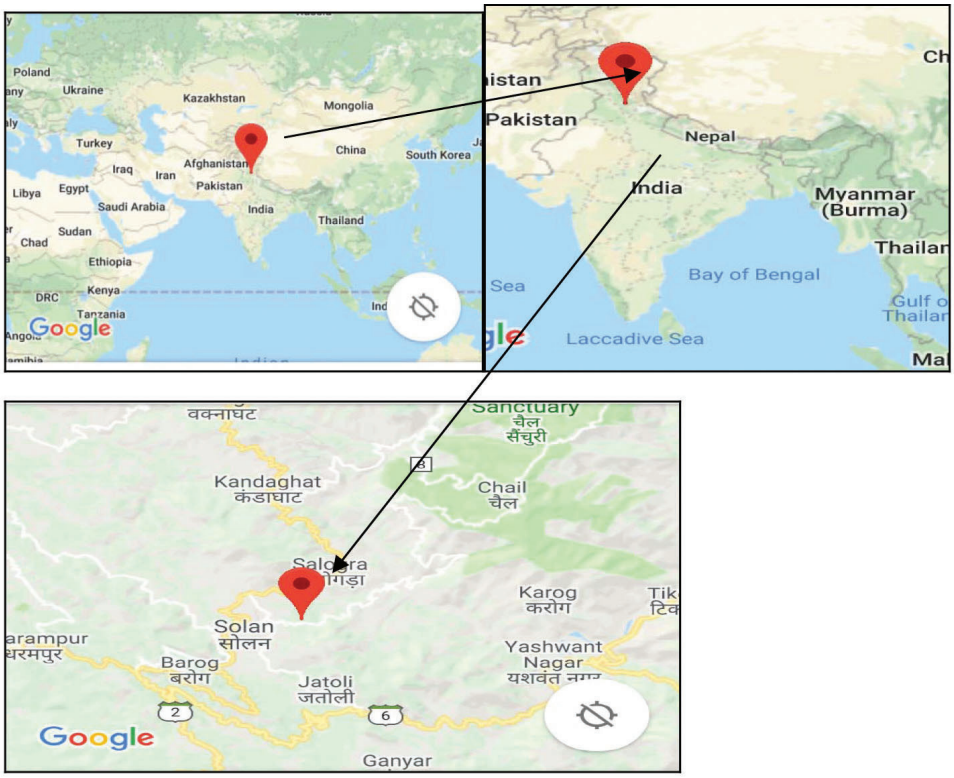

Fig. 1. Map showing the site of procurement of the plant material

Results and Discussion

Efficient regeneration under *in vitro*

Surface sterilized leaf explants were cultured on solid MS medium supplemented with 3.0 mg/l BA, 1.0 mg/l Kinetin and 0.5 mg/l NAA results inmaximum 84.26% establishment as shown in Table 1 (Figure 2 A-B). The frequency of shoots regeneration varied according to the concentration and combination of BA, Kinetin and NAA. This may suggest that synergistic effect of BA and NAA increased shoot morphogenesis which was also reported in other plants (Amuth *et al*., 2003; Lee and Pijut, 2017). The microshoots obtained from established leaf explants were transferred to multiplication medium. Highest *in vitro* multiplication and proliferation of microshoots observed in MS medium comprising 1.00 mg/l BA, 0.50 mg/l Kinetin, 0.25 mg/l NAA and 1.00 mg/l GA_3 which was further selected and used for the multiplication and proliferation of microshoots (Table 2) (Figure 2C). Similarly, the effect of these parameters on the regeneration potential have also been reported earlier in *Swertia* and other plant species (Thind *et al*., 2008; Shailja *et al*., 2017). Therefore, induction of direct ad-

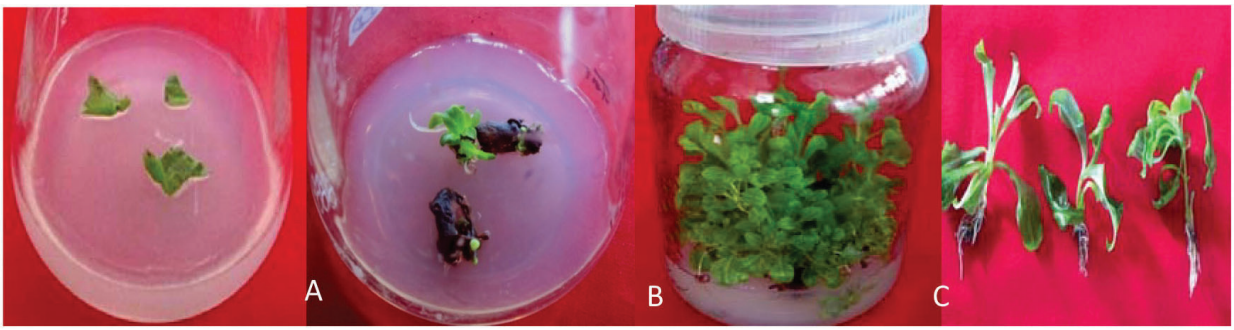

Fig. 2A. Inoculated leaf explants on solid MS medium supplemented with 3.0 mg/l BA + 1.0 mg/l Kinetin + 0.5 mg/l NAA **B.** Direct shoot induction after two weeks of incubation **C.** Shoot proliferation and elongation after four weeks **D.** Rooted plantlets

Table 1. Effect of different concentrations of BA, Kinetin and NAA on direct regeneration of microshoots from leaf explants after four weeks of incubation

BA (mg/l)	Kinetin (mg/l)	NAA (mg/l)	[1,2]Percent shoot induction	[2]Average number of shoots per explant	[2]Average shoot length (cm)
0.0	0.0	0.0	0.00 (0.00)^{k}	0.00^{g}	0.00^{h}
1.0	1.0	0.5	68.91 (56.12)^{f}	5.26^{b,c,d,e,f}	2.41^{c,d,e,f,g}
2.0	1.0	0.5	81.98 (65.07)^{b}	7.60^{a,b}	4.20^{a,b}
3.0	1.0	0.5	84.26 (68.07)^{a}	8.17^{a}	4.34^{a}
4.0	1.0	0.5	80.55 (63.84)^{c}	7.26^{a,b,c}	3.44^{b,c,d}
1.0	2.0	0.5	71.68 (59.46)^{d,e}	6.84^{a,b,c,d}	3.34^{b,c,d,e}
2.0	2.0	0.5	74.38 (59.60)^{d,e}	6.92^{a,b,c}	3.46^{b,c,d}
3.0	2.0	0.5	76.76 (61.19)^{d}	6.98^{a,b,c}	3.47^{b,c}
4.0	2.0	0.5	75.37 (60.26)^{d,e}	6.57^{a,b,c,d,e}	2.96^{c,d,e,f}
1.0	1.0	1.0	74.17 (59.46)^{d,e}	5.88^{a,b,c,d,e,f}	2.97^{c,d,e,f}
2.0	1.0	1.0	69.99 (56.78)^{f}	5.52^{b,c,d,e,f}	2.56^{c,d,e,f,g}
3.0	1.0	1.0	65.57 (54.07)^{g,h}	5.26^{b,c,d,e,f}	2.31^{c,d,e,f,g}
4.0	1.0	1.0	64.84 (53.63)^{h}	5.04^{c,d,e,f}	2.22^{d,e,f,g}
1.0	2.0	1.0	64.18 (53.24)^{h}	4.51^{d,e,f}	2.11^{e,f,g}
2.0	2.0	1.0	62.64 (52.32)^{h}	4.44^{e,f}	2.08^{f,g}
3.0	2.0	1.0	58.59 (49.95)^{i}	3.94^{f}	1.99^{f,g}
4.0	2.0	1.0	54.39 (47.52)^{j}	3.96^{f}	1.5^{g}
C.D.			2.26	2.03	1.06
S.E.±			1.10	0.99	0.52

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

ventitious shoots without intermediate stage of callusing provide a rapid and dependable method for the production of large quantity of uniform plantlets. But to get the maximum proliferation, shoots were progressively subculturedafter every four weeks and the increase in proliferation resulted was so, as continuous subculturingleads to rejuvenate the adult tissues (Deora and Shekhawat, 1995; Kumari and Kanwar, 2020).

In vitro root induction

After the proliferation, 4.00-4.05 cm shoots were separated from the medium and transferred to rooting medium further. Control (half strength MS basal medium) without activated charcoal showed no response to *in vitro* rooting experiments (Table 3). Half strength MS medium supplemented with only activated charcoal also did not respond for rooting effect. IBA found to be a potent root inducing hormone (Ahuja *et al*., 2003; Kumari and Kanwar, 2020). From the data presented it was noticed microshoots dipped in 1.0 mg/l IBA for 24 hours and then transferred in medium comprised of half strength MS basal supplemented with 0.02% activated charcoal results in 75.21% rooting with 2.06 number of roots having 2.23 cm length, as shown in Figure 2 (D). Medium supplemented with charcoal made good effect on root growth providing no callus growth which helps in the better plant survival during hardening stage.Profuse roots were observed on half strength MS medium supplemented with 9.85µM IBA with 2% sucrose after 20-25 days of culturing by Chalageri and Babu (2012). Thus, from above results it was seen that pretreatment with auxins increases *in vitro* root inductionand activated charcoal helps better root growth at all its concentration tried but it gave much better results at its lower concentration than higher concentration.

Hardening and acclimatization of *in vitro* raised plantlets

Further, suitability of different combinations of the hardening mixtures as mentioned in Table 4 was studied for the percent survival of directly raised rooted plantlets. It may be noted that percent survival of 52.28% insand+cocopeat+perlitepotting mixture obtained was statistically significant as compared other potting mixture. Therefore, mixture of sand, cocopeat and perlite may found to be more suitable hardening mixture for the acclimatization of *in vitro* raised plantlets and thus also selected for the further acclimatization of plantlets. Similarly, Balaraju *et al*. (2011) in their study transferred regenerated plants of *Swertia* into different substrates under controlled conditions for hardening of the plants. Effect of hardening at weekly intervals on

Table 2. Effect of different concentrations and combinations of BA, Kinetin, NAA and GA_3on shoot proliferation of directly regenerated shoots after four weeks of incubation

Growth Regulators (mg/l)				[1,2]Shoot proliferation (%)	[2]Average number of shoots per explant	[2]Average shoot length (cm)
BA	Kinetin	NAA	GA_3			
0.00	0.0	0.00	0.00	0.00(0.00)^{h}	0.00^{e}	0.00^{k}
1.00	0.25	0.25	1.00	60.92(51.31)^{f}	5.82^{a}	4.33^{b}
1.00	0.50	0.25	1.00	88.91(70.60)^{a}	6.14^{a}	6.12^{a}
1.00	0.75	0.25	1.00	87.87(69.61)^{a,b}	5.64^{a}	5.86^{b}
1.00	1.00	0.25	1.00	86.20(68.19)^{b,c}	4.23^{b}	5.62^{c}
2.00	0.25	0.25	1.00	84.47(66.80)^{c}	4.16^{b}	5.44^{d}
2.00	0.50	0.25	1.00	75.58(60.39)^{d}	3.53^{c}	5.23^{e}
2.00	0.75	0.25	1.00	68.10(55.61)^{e}	3.61^{c}	4.34^{f}
2.00	1.00	0.25	1.00	62.26(52.10)^{f}	3.43^{c}	3.28^{g}
3.00	0.25	0.25	1.00	60.20(50.89)^{f}	3.22^{c,d}	2.24^{h}
3.00	0.50	0.25	1.00	58.68(50.00)^{f}	2.86^{d}	1.36^{i}
3.00	0.75	0.25	1.00	54.44(47.55)^{g}	2.66^{d}	1.08^{j}
3.00	1.00	0.25	1.00	52.53(46.45)^{g}	2.65^{d}	1.06^{j}
C.D.				1.93	0.38	0.02
S.E.±				0.95	0.19	0.01

[1]Figures in parentheses are arc sine transformed values

[2]Means followed by different letters are significantly different at *P*=0.05 according to Duncan's multiple range test

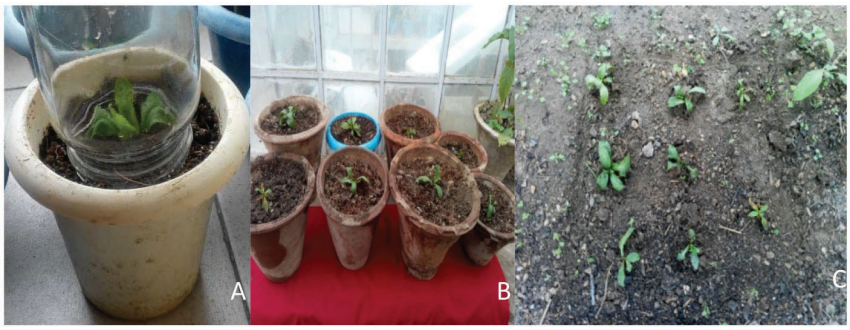

Fig. 3A. Hardened plant after one month of transfer **B** Hardened plants after four months **C** Completely hardened plants in field

morphological parameters of plantlets when noted it was observed during 28th week, maximum plant height 8.89 cm and maximum number of leaves per plant 12.08 observed. Internodal distance increased with weekly interval and found maximum in hardened plantlets of 28th week. Limited literature on the hardening of *Swertia* plantlets regenerated through direct organogenesisis present as compare to indirect organogenesis. Roots developed by excised shoots of *Swertia chirayita* were viable and plantlets were successfully acclimatized to *ex vitro* conditions (Figure 3 A-C).

Conclusion

Swertia chirayita an endangered plant being exploited at larger scale so there should be an alternative to provide the sufficient material as per demand

Table 4. Effect of hardening mixtures on acclimatization of *in vitro* raised plantlets after four weeks of incubation

Substrate	[1]Percent survival directly raised plantlets
Sand	0.00(0.00)[c]
Soil	0.00(0.00)[c]
Cocopeat+Perlite	0.00(0.00)[c]
Sand+Cocopeat+Perlite	52.26(46.29)[a]
Soil+Cocopeat	0.00(0.00)[c]
Sand+Soil+Cocopeat+Perlite	20.16(26.67)[b]
C.D.	0.02
S.E.±	0.01

[1]Figures in parentheses are arc sine transformed values

Table 3. Effect of different concentrations of IBA and activated charcoal on *in vitro* root induction from directly regenerated microshoots after four weeks of incubation

Medium	Dipping 24 hours (IBA) mg/l	Activated charcoal (%)	[1, 2]Percent rooting	[2]Number of roots	[2]Root length (cm)	Callusing -
MS basal	-	-	0.00(0.00)g	0.00i	0.00d	-
MS basal	-	0.02	0.00(0.00)g	0.00i	0.00d	-
½ MS basal	-	0.04	0.00(0.00)g	0.00i	0.00d	-
½ MS basal	1.0	-	72.43(58.33)a,b	2.12b	2.16a	-
½ MS basal	2.0	-	69.62(56.55)b,c	1.56f	1.88a,b	-
½ MS basal	3.0	-	57.69(49.42)e	1.42g	1.56b	-
½ MS basal	1.0	0.02	75.21(60.14)a	2.23a	2.06a	-
½ MS basal	2.0	0.02	70.35(57.01)b	2.08c	1.98a,b	-
½ MS basal	3.0	0.02	64.45(53.40)d	1.88e	1.76a,b	-
½ MS basal	1.0	0.04	66.61(54.71)c,d	1.95d	1.85a,b	-
½ MS basal	2.0	0.04	60.18(50.87)e	1.56f	1.55b	
½ MS basal	3.0	0.04	45.54(42.43)f	1.33h	0.38c	
C.D.			1.90	0.02	0.62	
S.E.±			0.94	0.01	0.31	

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test
(-: No callus)

and conserve the species. In our study, protocol for the effective adventitious shoot regeneration was standardized to obtained the maximum number of genetically uniform plants of high pharmaceutical value. Shoots were induced from immature leaf explants taken from field grown plantswhich reduced the cost and time for rgeneration. Acclimatized plants possess normal vegetative and reproductive development. The method standardized in the present study allow a massive production of *Swertia chirayita* plants for further pharmacological studies. As it is the preliminary study, where focus was to develop a reliable and effective protocol of direct method of micropropagationon which all further studies based, the research can be developed further to be used in molecular studies and in genetic transformation studies of *Swertiachirayita* in further works.

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Disclosure statement

Conflict of interest:Authors declare no conflict of interest.

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