De novo Shoot Organogenesis from Cultured Root Explants of *Swertia chirata* Buch.-Ham.ex Wall.: An Endangered Medicinal Plant.

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Abstract: *In vitro* regeneration of plants from root cultures of *Swertia chirata* Buch.-Ham. ex Wall was obtained. Root explants from axenic shoot cultures were used for shoot induction. Optimal shoot regeneration without any callus intervention was observed on 1/2 MS (Murashige and Skoog's medium, 1962) + 4.44 µM 6, benzylaminopurine (BAP) +1.07 µM α-naphthalene acetic acid (NAA).Regenerated shoots were further multiplied on full strength MS medium supplemented with different concentrations of plant growth regulators. Maximum shoot multiplication was achieved on MS medium fortified with 4.44 µM BAP + 2.85 µM indole-3 acetic acid (IAA) + 271.45 µM adenine sulphate (Ads). Best results of rooting were observed on 1/2 strength MS medium containing 4.90 µM IBA. Plants with well-developed shoots and roots were successfully hardened with over 80% survivability.
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Keywords: *Swertia chirata*, root culture, *de novo* organogenesis, *in vitro* plant regeneration.

Abbreviations: MS: Murashige and Skoog (1962); **BAP:** 6, Benzylaminopurine; **Kn:** 6-Furfurylaminopurine; **NAA:** α-Naphthalene Acetic Acid; **IBA:** Indole-3 butyric acid; **IAA**: Indole-3 acetic acid; **GA_3**: Gibberellic acid; **Ads:** Adenine sulphate; **PGR:** Plant growth regulator

Introduction

Swertia chirata Buch.- Ham.- commonly known as chirayata is a medicinal plant of family Gentianaceae is native to temperate Himalaya (altitude above 4,000-10,000 ft.). The plant has an erect, about 2-3 ft long stem and the whole plant is bitter in taste. *S.chirata* contains a yellow bitter ophelic acid and two bitter glucosides chiratin and amarogentin. The root is said to be the most powerful part which is included in the British and American Pharmacopoeias and is used against a variety of diseases. The bitterness, antihelmintic, hypoglycemic and antipyretic properties are attributed to amarogentin ; most bitter compound isolated till date (Karan et al., 1996), swerchirin, swertiamarin and other active principles of the herb. The whole plant contains gentiamine alkaloids and the aerial part contains xanthones. Herbal medicines such as Ayush-64, Diabecon, Mensturyl syrup and Melicon ointment contain chirata extract in different amounts (Edwin et al., 1988, Valecha et al., 2000 and Mitra et al., 1996). The drug obtained from the dried plant is held in high esteem in India and is prescribed in a variety of forms and combinations in chronic fever, anemia and is used as special remedy for bronchial asthma, liver and stomach disorders, malaria and diabetes.

Several species of the genus *Swertia* are available in India and other countries and used as adulterant. However, *S.chirata* from India *and S.japonica* and *S.pseudochinenesis* from Japan are considered elite species because of their medicinal value and revenue-generating potential.

Chirayata is difficult to propagate on mass scale via seed owing to non-availability of seeds or due to harvesting of plants before seeds mature. The species is, therefore, deprived of natural regeneration. Also, the low viability and germination percentage of seeds and the necessary delicate field handling of the seedlings are some of the factors that discourage agro technology development and commercial cultivation of the species. There is an increasing pharmaceutical demand of the species both, in the indigenous and the world market but lack of large-scale commercial plantations of the herb continues. Destructive harvesting and loss of habitat has diminished the existing populations of *S.chirata*. The species is among the highly prioritized medicinal plants of India as identified by National Medicinal Plant Board, Govt. Of India and according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria, this priority plant has been designated as critically endangered .The need to develop techniques for its mass multiplication through different regeneration pathways thus, becomes imperative.

Documented literature reveals that there are limited reports on *in vitro* propagation of *Swertia chirata.* Micropropagation via field-grown nodal explants has been reported by Ahuja *et al.* (2003), Chaudhuri *et al.* (2007), Koul *et al*. (2009) and Pant *et al.,* 2010. Joshi and Dhawan (2007 a) and Balaraju *et al.* (2009) published reports on *in vitro* propagation of *S. chirata* using shoot tip explants derived from *in vitro* grown seedlings. ISSR marker analysis of genetic diversity among *S.chirata* genotypes of temperate Himalayan region of India was done by Joshi and Dhawan (2007 b). Chaudhuri *et al.* 2008 and 2009 reported direct shoot regeneration from *in vitro* leaves regeneration via immature seed cultures of *S. chirata*. Wang *et al.* (2009) described *in vitro* shoot regeneration from leaves taken from field-grown plants.

Root tissue has been proven to be highly regenerative explant and is relatively easy to maintain and manipulate *in vitro* (Vincour *et al.*, 2000; Franklin, 2004). Besides, shoots developing on root segments of the same plant have been reported to be genetically uniform and the method, an alternative way for clonal propagation. (Ostazeski and Henson, 1965; Budd, 1973; Chaturvedi *et al.*, 1981; Sharma *et al.*, 1993). The only available report on culture of root segments of *S.chirata* procured from *in vitro* raised seedlings was by Wawrosch *et al.* (1999). However, low frequency of shoot regeneration and hyperhydration of shoots hindered the production of quality shoots employing the procedure described by them. The present report communicates a reproducible protocol for regeneration of well-developed and healthy *S. chirata* plantlets via root segment culture without any intervening callus phase. The findings will be useful in conservation and mass multiplication of this endangered medicinal plant.

Material and Methods

Explant Source

In our previous study on *S.chirata* (Pant *et al.,* 2010) the shoots were induced from nodal explants. Regenerated shoots were further multiplied , subcultured and maintained on full strength MS medium supplemented with BAP(4.44 µM). Shoots having 1 or 2 nodes were rooted on half-strength MS medium supplemented with 4.90 µM indole-3 butyric acid (IBA).Ten – weeks old *in vitro* developed roots were used as an explant material for direct shoot organogenesis. For this, *in vitro* regenerated shoots via nodal explants were rooted on half-strength MS medium supplemented with 2% sucrose and IBA (4.90 µM). Aseptically removed long roots were repeatedly washed in sterile distilled water to free them from agar adhering to the surface. Subsequently they were dissected into 2-3 cm long proximal, middle and distal segments and placed horizontally on the culture medium for shoot organogenesis.

In vitro Shoot Organogenesis

Half-strength MS medium solidified with 0.7% agar (w/v) and supplemented with 2% sucrose and varying concentrations and combinations of phytohormones viz BAP(2.22 µM-22.20 µM) and NAA(1.07 µM- 5.37 µM) was tested for direct shoot regeneration from axenic root cultures. MS medium lacking plant growth regulators served as control. Data on average shoot number per explant and average shoot length (cm) was recorded after an interval of 4 weeks.

In vitro Shoot Multiplication

Once the culture conditions for optimum shoot induction from root explants were established, the proliferated shoots were subcultured onto fresh medium of same composition to establish an initial stock of shoots. These shoots were further used to assess the effect of growth regulators on further *in vitro* shoot multiplication. Full strength MS medium agarified with 0.7% agar (w/v) and fortified with 3% sucrose and varying concentrations and combinations of plant growth regulators were used for the purpose. Experiments conducted to obtain maximum shoot multiplication largely consisted of experiments pertaining to cytokinin (4.44 µM – 13.32 µM BAP) alone and in combination with auxin (1.14 µM – 2.85 µM IAA) and/or adjuvant adenine sulphate (271.45 µM). Observations pertaining to average shoot number and average shoot length were recorded after periodic intervals of 4 weeks and 8 weeks. Routine sub culturing of multiple shoots was carried out at an interval of every 3-week.

In vitro Rooting

Experiments on *in vitro* rooting of *in vitro* grown shoots were attempted with 2-3 cm long shoots produced during shoot multiplication. Shoots were cultured in each tube on MS medium containing 2% sucrose and supplemented with auxins IAA (1.14 µM - 11.40 µM), IBA (0.98 µM - 9.80 µM) and NAA (1.07 µM - 10.74 µM). Single shoots were cultured for initiation of roots and observations were recorded after 4 weeks and 8 weeks. Rooting response was recorded in terms of average number of roots produced and average root length.

Medium and Culture conditions

The pH of medium was adjusted to 5.8 before adding 0.7% agar (w/v) and sterilized by autoclaving at 15lbs (1.8 kg/cm^2) pressure at 121^{0}C for 15 minutes. Cultures were incubated at 25 ± 1^{0}C

for 16 hours in light (illuminated by 40 watt fluorescent tubes, 50µE m^{-2} s^{-1}) and for 8 hours in dark cycle irradiance by cool fluorescent tubes.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to liquid 1/4 strength MS strength medium having 2% sucrose and devoid of any plant growth regulator, for 7 days in flasks. Plantlets with well- developed roots were then removed from culture flasks and the roots gently washed under running tap water. They were then transferred to polybags containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags and maintained in high-density double check agronet open shade house. The plantlets were suitably irrigated with tap water .The polythene bags were periodically withdrawn to acclimatize the plantlets and when new leaves started emerging the polybags were completely removed. Hardened plantlets were subsequently transferred to soil in pots for further growth and development.

Statistical Analysis

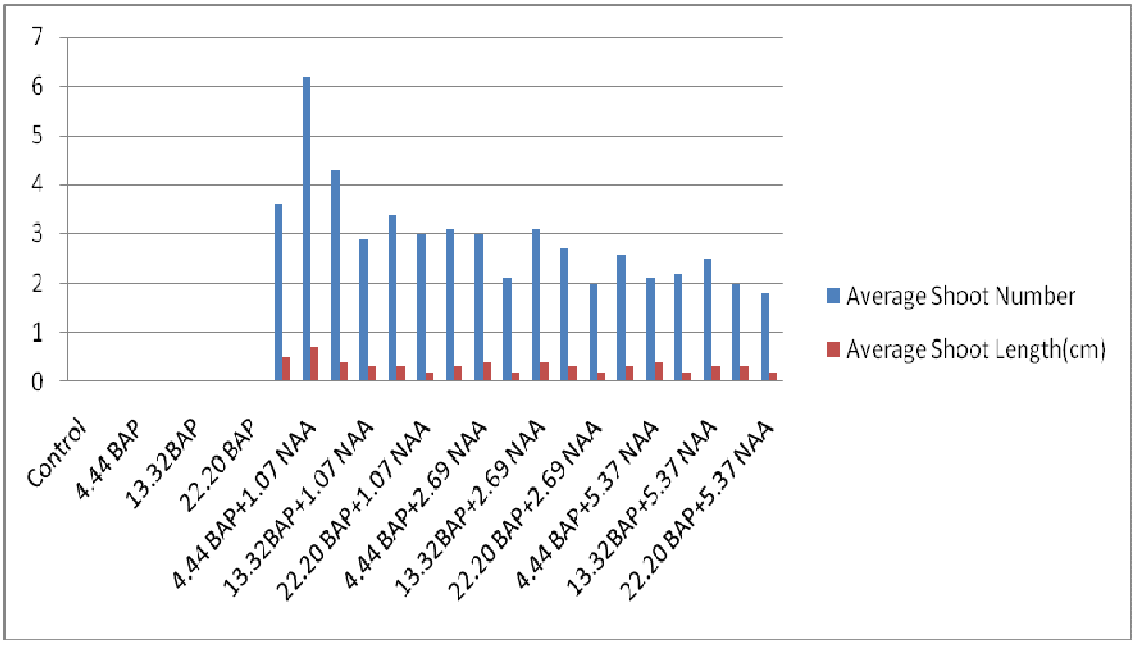
Experiments were done in triplicate and each treatment consisted of minimum ten replicates. The data was analysed using analysis of variance (ANOVA) of Completely randomized design (CRD) in GenStat Discovery Edition 13.The significance level was determined at P < = 0.05.Mean values of treatments were compared with Least Significance Difference (LSD).

Results:

***In vitro* shoot organogenesis**

De novo shoot organogenesis occurred only on proximal or middle sections of the roots. Distal regions of root explants proved to be incompetent for the purpose. Among the different treatments tried for shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) was found to be most optimal giving an average of 6.7 shoots per explant with mean shoot length of 0.7 cm without any growth of lateral roots within a period of 4 weeks [Figure 1]. MS medium without any plant growth regulator or augmented with BAP alone proved to be ineffective for shoot regeneration. On increasing the concentration of NAA to 2.69 µM and 5.37 µM , an enhanced induction of lateral roots was observed with a marked decrease in shoot bud induction from explants.(Graph 1).

Graph 1: Effect of PGR (µM) Treatment on Adventitious Shoot Regeneration from Root Explants



***In vitro* shoot multiplication**

The media combinations reported to be most optimal in our previous study on *S.chirata* (Pant *et al*., 2010) were MS medium supplemented with different concentrations of BAP, IAA and /or Ads for shoot multiplication and half-strength MS containing IBA for subsequent rooting. Similar combinations were tried in the present study and favourable results were obtained. On transferring *in vitro* grown shoots onto multiplication media, all media combinations tested were capable of inducing multiplication within 2-3 wks. Final observations in terms of mean number of shoots and shoot length were recorded after a period of 4 weeks and 8 weeks. All the treatments were significantly superior to the control except the treatments C4 and C6. In C4 (MS + 13.32 µM BAP) the average shoot number after 4 weeks and in C6 [MS+ 13.32 µM BAP+ 2.85 µM IAA] the average shoot length after 8 weeks was at par with the control. Amongst all, treatment C8 was found to be the best in all parameters. In this combination(MS + 4.44 µM BAP + 2.85 µM IAA + 271.45µM Ads) an average of 10.0 number of shoots with shoot length 2.0 cm after 4 weeks and mean shoot number 18.7 with shoot length 4.0 cm after 8 weeks was recorded [Table1] [Figure 3] .

Table 1: Effect of Plant Growth Regulators on *In vitro* Multiplication of Regenerated Microshoots

Treatment	MS medium +PGR(µM)			Average number of shoots		Average length of shoots(cm)	
	BAP	IAA	Ads	After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
C1	0.0	0.0	0.0	3.5	4	0.7	1.0
C2	4.44	0.0	0.0	4.4	7.4	1.6	2.3
C3	8.88	0.0	0.0	5.4	15	2.3	3.3
C4	13.32	0.0	0.0	5.3	9.1	1.2	1.7
C5	4.44	2.85	0.0	7.3	10.7	1.2	1.8
C6	8.88	2.85	0.0	6.4	9.8	1.1	1.6
C7	13.32	2.85	0.0	6.3	12.4	1.3	2.4
C8	**4.44**	**2.85**	**271.45**	**10.0**	**18.7**	**2.0**	**4.0**
C9	8.88	2.85	271.45	7.4	13.4	1.7	4.6
C10	13.32	2.85	271.45	8.2	11.5	1.9	2.7
Mean				6.4	11.2	1.5	2.5
LSD				1.8	2.8	0.4	1.8

After standardizing the media combination for shoot multiplication, *in vitro* multiplied shoots were subcultured on MS medium fortified with 4.44 µM BAP for maintenance of cultures.

***In vitro* rooting**

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama 1970). For the purpose, regenerated shoots were inoculated onto rooting media. It was observed that root primordia emerged from the shoot base within 10 to 14 days after shoot inoculation in all the treatments. Observations regarding mean root number and root length were recorded after 4 weeks and 8 weeks. All treatments were significantly superior to control. Optimal response was observed in the treatment R4(4.90 µM IBA) with average number of 21.0 roots per shoot after 4-weeks, 28.9 after 10-weeks and average root length 0.9 cm after 4-weeks & 1.6 cm after 10-weeks was observed in [Table 2] [Figure 4].

Table 2: Effect of Different Concentrations of IBA on Rooting of *In-vitro* Regenerated Shoots.

Treatment	1/2 strength MS medium +IBA (µM)	Average number of roots		Average length of roots(cm)	
		After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
R1	0.0	1.6	1.8	0.2	0.2
R2	0.98	6.2	12.1	0.6	1.1
R3	2.46	7.1	13.5	0.8	1.3
R4	**4.90**	**21.0**	**28.9**	**0.9**	**1.6**
R5	9.80	14.0	20.7	0.6	1.1
Mean		10.0	15.4	0.6	1.1
LSD		5.4	6.2	0.3	0.3

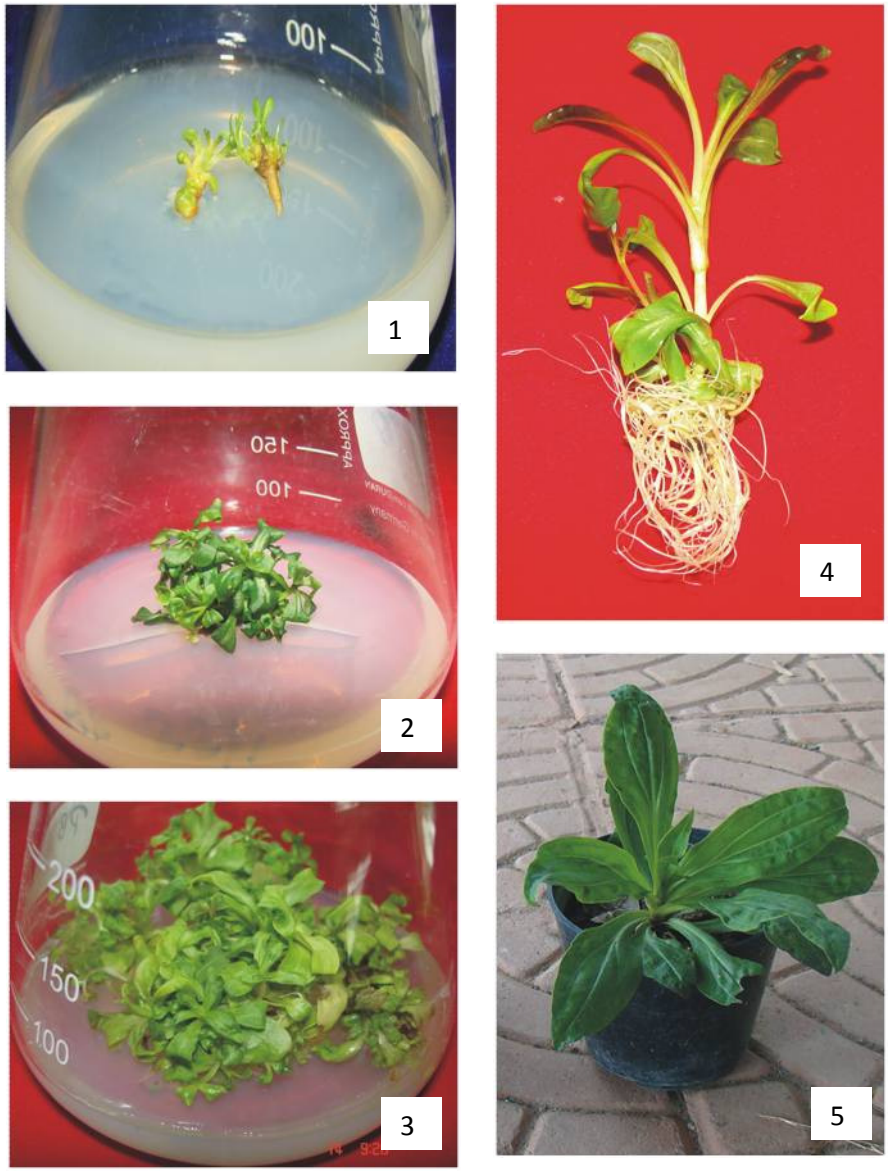


Fig. 1-5 Direct organogenesis of *Swertia chirata* via root explant:
(1) Shoot induction on root segments (2) Culture establishment
(3) Multiplication of shoots (4) *In vitro* rooted plantlet (5) Hardened plantlet.

Hardening and acclimatization

In-vitro hardening was done on liquid 1/4MS medium having 2% sucrose, for 7 days followed by acclimatization in agronet shade house in polybags containing a mixture of soil: sand :manure (1:1:1) and covered with perforated polythene bags. These polythene bags were withdrawn periodically and on emergence of new leaves, the polybags were completely removed. The plants maintained in shade house were irrigated with water. For next one month,

plants were maintained in net house. Well-acclimatized plants were shifted to pots containing soil and gave percentage survival of over 80%. [Figure 5].

Discussion

Careful observations revealed that of the different root segments tried for shoot bud initiation in our study, distal root segments remained non-responsive to shoot bud initiation while proximal and middle segments exhibited shoot regeneration and apparently no difference in the frequency and mode of shoot bud differentiation. Bud initiation and its formation uniformly from the proximal or middle portion of the root indicate that some secondary growth may also be essential for shoot initiation. The results are in corroboration with reports on other species where only the proximal and middle root sections exhibited *de novo* shoot organogenesis in *Comptonia peregrine* (Goforth and Torrey,1977), *Brassica napus* (Sharma and Thorpe, 1987), *Holostemma annulare* (Sudha *et al.*, 2000), *Populus tremula* (Vinocur *et al*.,2000), *Hypericum perforatum* (Zobayed and Saxena, 2003).

In the present study, among the different treatments tried for *in vitro* shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) gave maximum number of shoots without any growth of lateral roots within a period of 3 weeks . A synergistic effect of BAP and NAA has been highlighted in previous studies on shoot initiation from root explants of *Dalbergia sissoo* (Mukhopadhay and Mohan Ram,1981); *Citrus sinensis* (Burger and Hackett, 1986); *Citrus mitis* (Sim *et al.*, 1989); *Averrhoa carambola* (Kantharajah *et al.*,1992); *Clitoria ternatea* (Shahzad *et al.,*2007) and *Podophyllum hexandrum* (Chakraborty *et al*. ,2010) .

However, in our study, on increasing the concentration of NAA to 2.69 µM and 5.37 µM, significant reduction in shoot bud initiation was observed with an enhanced induction of lateral roots from cut end of roots. Root explants in our study were taken from *in vitro* regenerated roots growing in auxin-rich medium. On placing the explants in another auxin- rich medium may be an inhibiting factor for shoot bud differentiation while facilitating lateral root formation. This is also supported by the view that relatively high concentration of auxin suppresses the inception of shoot buds and promotes growth of lateral roots (Peterson, 1975). Cytokinins are considered to stimulate bud formation and inhibit lateral root formation. There are reports where BAP alone has found to be most efficient for shoot bud regeneration from root tissues in *Citrus mitis* (Sim *et al.,* 1989); *Citrus aurantifolia* (Bhat *et al.*, 1992); *Holostemma annulare* (Sudha *et al*., 2000); *Garnicia indica* (Deodhar *et al.*, 2000); *Blackstonia perfoliata* (Bijelovic *et al*. ,2004); *Crataeva nurvula* (Walia *et al.*,2004); *Melia azedarach* (Vila *et al.*, 2005). Contrastingly, in our study BAP when tried alone at different concentrations was found to be completely inefficient for shoot regeneration.

Wawrosch *et al.,* 1999 reported adventitious shoot regeneration from root explants of *S. chirata* where radicle explants from 3-week-old aseptically grown seedlings were used as primary explants for shoot organogenesis. They optimized a procedure of 3 weeks culturing root explants on BAP(3µM) supplemented MS medium followed by transfer to basal MS medium for another 3 weeks. However, the regeneration procedure described was not very efficient as only 1.9 shoots per explant could be achieved and the regenerated shoots were hyperhydrated. We report a significant improvement over these results as an average of 6.7 shoots per root explants could be obtained in our study which were further multiplied on MS medium supplemented with BAP, IAA and Ads and 18.0 fold multiplication was achieved. The regenerated shoots were healthy and robust without any observation of hyperhydration.

Rooting experiments were initiated after 180 days of subculturing to get maximum material for trying different hormone concentrations. Half-strength MS medium supplemented with IBA at 4.90 µM concentration proved to be most optimal for rooting. The results were similar to our previous findings on *in vitro* propagation of *S. chirata* via nodal explants. Wawrosch *et al.*(1999) had reported callus formation on exposure of shoots to media supplemented with upto 10 µM auxins for rooting. Therefore, they described a method of 2second dipping of shoots in an aqueous solution of 15 ppm NAA followed by 3-4 week cultivation period on hormone-free, half-strength MS medium for root development. In contrast, callusing did not occur in any of the treatments tried for *in vitro* rooting in our study and long, healthy root system developed on IBA augmented medium.

In vitro raised plants are heterotrophic in their mode of nutrition and cannot withstand the environmental conditions without proper hardening and acclimatization, thus, need to be hardened and acclimatized before field transplantation. Regenerated plantlets were hardened under controlled conditions of culture room on ¼ liquid MS medium and sucrose supply was decreased to 2%. After a period of one week, these plantlets were transferred to a rooting mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags in shade. Holes were made in polythene bags for gaseous

exchange. For acclimatizing the plantlets, bags were withdrawn periodically. During the process, the shoots elongated, leaves turned greener and expanded after which the polybags were completely removed. Hardened plants were subsequently transferred to soil filled in pots and over 80 % survivability was recorded.

We suggest that this ability of *S.chirata* plants of direct shoot organogenesis via root in nature, juvenile tissues of the root and specific combinations of growth regulators collectively triggered the process of direct shoot differentiation from root explants *in vitro*.

Conclusion

This study has revealed the morphogenetic potential of *in vitro* grown roots of *S.chirata* as a source tissue for micropropagation. The explants can be easily and regularly obtained from established shoot cultures and do not require disinfection treatment hence being ideal for germplasm exchange and cryopreservation. Normal root culture establishment for a number of plant species have the ability to accumulate secondary products similar to those found in mother donor plant (Tabata *et al.*, 1972; Whitten *et al.,* 1981). In *S.chirata* root being the most important part of the plant for medicinal purposes holds importance as the starting material for tissue culture studies. The present protocol will therefore serve as alternative means for *in vitro* clonal propagation of this endangered plant with immense medicinal value and further biochemical and physiological investigations.

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De novo Shoot Organogenesis from Cultured Root Explants of *Swertia chirata* Buch.-Ham.ex Wall.: An Endangered Medicinal Plant.

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Abstract: *In vitro* regeneration of plants from root cultures of *Swertia chirata* Buch.-Ham. ex Wall was obtained. Root explants from axenic shoot cultures were used for shoot induction. Optimal shoot regeneration without any callus intervention was observed on 1/2 MS (Murashige and Skoog's medium, 1962) + 4.44 µM 6, benzylaminopurine (BAP) +1.07 µM α-naphthalene acetic acid (NAA).Regenerated shoots were further multiplied on full strength MS medium supplemented with different concentrations of plant growth regulators. Maximum shoot multiplication was achieved on MS medium fortified with 4.44 µM BAP + 2.85 µM indole-3 acetic acid (IAA) + 271.45 µM adenine sulphate (Ads). Best results of rooting were observed on 1/2 strength MS medium containing 4.90 µM IBA. Plants with well-developed shoots and roots were successfully hardened with over 80% survivability.
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Keywords: *Swertia chirata*, root culture, *de novo* organogenesis, *in vitro* plant regeneration.

Abbreviations: MS: Murashige and Skoog (1962); **BAP:** 6, Benzylaminopurine; **Kn:** 6-Furfurylaminopurine; **NAA:** α-Naphthalene Acetic Acid; **IBA:** Indole-3 butyric acid; **IAA**: Indole-3 acetic acid; **GA_3**: Gibberellic acid; **Ads:** Adenine sulphate; **PGR:** Plant growth regulator

Introduction

Swertia chirata Buch.- Ham.- commonly known as chirayata is a medicinal plant of family Gentianaceae is native to temperate Himalaya (altitude above 4,000-10,000 ft.). The plant has an erect, about 2-3 ft long stem and the whole plant is bitter in taste. *S.chirata* contains a yellow bitter ophelic acid and two bitter glucosides chiratin and amarogentin. The root is said to be the most powerful part which is included in the British and American Pharmacopoeias and is used against a variety of diseases. The bitterness, antihelmintic, hypoglycemic and antipyretic properties are attributed to amarogentin ; most bitter compound isolated till date (Karan et al., 1996), swerchirin, swertiamarin and other active principles of the herb. The whole plant contains gentiamine alkaloids and the aerial part contains xanthones. Herbal medicines such as Ayush-64, Diabecon, Mensturyl syrup and Melicon ointment contain chirata extract in different amounts (Edwin et al., 1988, Valecha et al., 2000 and Mitra et al., 1996). The drug obtained from the dried plant is held in high esteem in India and is prescribed in a variety of forms and combinations in chronic fever, anemia and is used as special remedy for bronchial asthma, liver and stomach disorders, malaria and diabetes.

Several species of the genus *Swertia* are available in India and other countries and used as adulterant. However, *S.chirata* from India *and S.japonica* and *S.pseudochinenesis* from Japan are considered elite species because of their medicinal value and revenue-generating potential.

Chirayata is difficult to propagate on mass scale via seed owing to non-availability of seeds or due to harvesting of plants before seeds mature. The species is, therefore, deprived of natural regeneration. Also, the low viability and germination percentage of seeds and the necessary delicate field handling of the seedlings are some of the factors that discourage agro technology development and commercial cultivation of the species. There is an increasing pharmaceutical demand of the species both, in the indigenous and the world market but lack of large-scale commercial plantations of the herb continues. Destructive harvesting and loss of habitat has diminished the existing populations of *S.chirata*. The species is among the highly prioritized medicinal plants of India as identified by National Medicinal Plant Board, Govt. Of India and according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria, this priority plant has been designated as critically endangered .The need to develop techniques for its mass multiplication through different regeneration pathways thus, becomes imperative.

Documented literature reveals that there are limited reports on *in vitro* propagation of *Swertia chirata.* Micropropagation via field-grown nodal explants has been reported by Ahuja *et al.* (2003), Chaudhuri *et al.* (2007), Koul *et al*. (2009) and Pant *et al.,* 2010. Joshi and Dhawan (2007 a) and Balaraju *et al.* (2009) published reports on *in vitro* propagation of *S. chirata* using shoot tip explants derived from *in vitro* grown seedlings. ISSR marker analysis of genetic diversity among *S.chirata* genotypes of temperate Himalayan region of India was done by Joshi and Dhawan (2007 b). Chaudhuri *et al.* 2008 and 2009 reported direct shoot regeneration from *in vitro* leaves regeneration via immature seed cultures of *S. chirata*. Wang *et al.* (2009) described *in vitro* shoot regeneration from leaves taken from field-grown plants.

Root tissue has been proven to be highly regenerative explant and is relatively easy to maintain and manipulate *in vitro* (Vincour *et al.*, 2000; Franklin, 2004). Besides, shoots developing on root segments of the same plant have been reported to be genetically uniform and the method, an alternative way for clonal propagation. (Ostazeski and Henson, 1965; Budd, 1973; Chaturvedi *et al.*, 1981; Sharma *et al.*, 1993). The only available report on culture of root segments of *S.chirata* procured from *in vitro* raised seedlings was by Wawrosch *et al.* (1999). However, low frequency of shoot regeneration and hyperhydration of shoots hindered the production of quality shoots employing the procedure described by them. The present report communicates a reproducible protocol for regeneration of well-developed and healthy *S. chirata* plantlets via root segment culture without any intervening callus phase. The findings will be useful in conservation and mass multiplication of this endangered medicinal plant.

Material and Methods

Explant Source

In our previous study on *S.chirata* (Pant *et al.,* 2010) the shoots were induced from nodal explants. Regenerated shoots were further multiplied , subcultured and maintained on full strength MS medium supplemented with BAP(4.44 µM). Shoots having 1 or 2 nodes were rooted on half-strength MS medium supplemented with 4.90 µM indole-3 butyric acid (IBA).Ten – weeks old *in vitro* developed roots were used as an explant material for direct shoot organogenesis. For this, *in vitro* regenerated shoots via nodal explants were rooted on half-strength MS medium supplemented with 2% sucrose and IBA (4.90 µM). Aseptically removed long roots were repeatedly washed in sterile distilled water to free them from agar adhering to the surface. Subsequently they were dissected into 2-3 cm long proximal, middle and distal segments and placed horizontally on the culture medium for shoot organogenesis.

In vitro Shoot Organogenesis

Half-strength MS medium solidified with 0.7% agar (w/v) and supplemented with 2% sucrose and varying concentrations and combinations of phytohormones viz BAP(2.22 µM-22.20 µM) and NAA(1.07 µM- 5.37 µM) was tested for direct shoot regeneration from axenic root cultures. MS medium lacking plant growth regulators served as control. Data on average shoot number per explant and average shoot length (cm) was recorded after an interval of 4 weeks.

In vitro Shoot Multiplication

Once the culture conditions for optimum shoot induction from root explants were established, the proliferated shoots were subcultured onto fresh medium of same composition to establish an initial stock of shoots. These shoots were further used to assess the effect of growth regulators on further *in vitro* shoot multiplication. Full strength MS medium agarified with 0.7% agar (w/v) and fortified with 3% sucrose and varying concentrations and combinations of plant growth regulators were used for the purpose. Experiments conducted to obtain maximum shoot multiplication largely consisted of experiments pertaining to cytokinin (4.44 µM – 13.32 µM BAP) alone and in combination with auxin (1.14 µM – 2.85 µM IAA) and/or adjuvant adenine sulphate (271.45 µM). Observations pertaining to average shoot number and average shoot length were recorded after periodic intervals of 4 weeks and 8 weeks. Routine sub culturing of multiple shoots was carried out at an interval of every 3-week.

In vitro Rooting

Experiments on *in vitro* rooting of *in vitro* grown shoots were attempted with 2-3 cm long shoots produced during shoot multiplication. Shoots were cultured in each tube on MS medium containing 2% sucrose and supplemented with auxins IAA (1.14 µM - 11.40 µM), IBA (0.98 µM - 9.80 µM) and NAA (1.07 µM - 10.74 µM). Single shoots were cultured for initiation of roots and observations were recorded after 4 weeks and 8 weeks. Rooting response was recorded in terms of average number of roots produced and average root length.

Medium and Culture conditions

The pH of medium was adjusted to 5.8 before adding 0.7% agar (w/v) and sterilized by autoclaving at 15lbs (1.8 kg/cm^2) pressure at 121^{0}C for 15 minutes. Cultures were incubated at 25 ± 1^{0}C

for 16 hours in light (illuminated by 40 watt fluorescent tubes, 50µE m^{-2} s^{-1}) and for 8 hours in dark cycle irradiance by cool fluorescent tubes.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to liquid 1/4 strength MS strength medium having 2% sucrose and devoid of any plant growth regulator, for 7 days in flasks. Plantlets with well- developed roots were then removed from culture flasks and the roots gently washed under running tap water. They were then transferred to polybags containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags and maintained in high-density double check agronet open shade house. The plantlets were suitably irrigated with tap water .The polythene bags were periodically withdrawn to acclimatize the plantlets and when new leaves started emerging the polybags were completely removed. Hardened plantlets were subsequently transferred to soil in pots for further growth and development.

Statistical Analysis

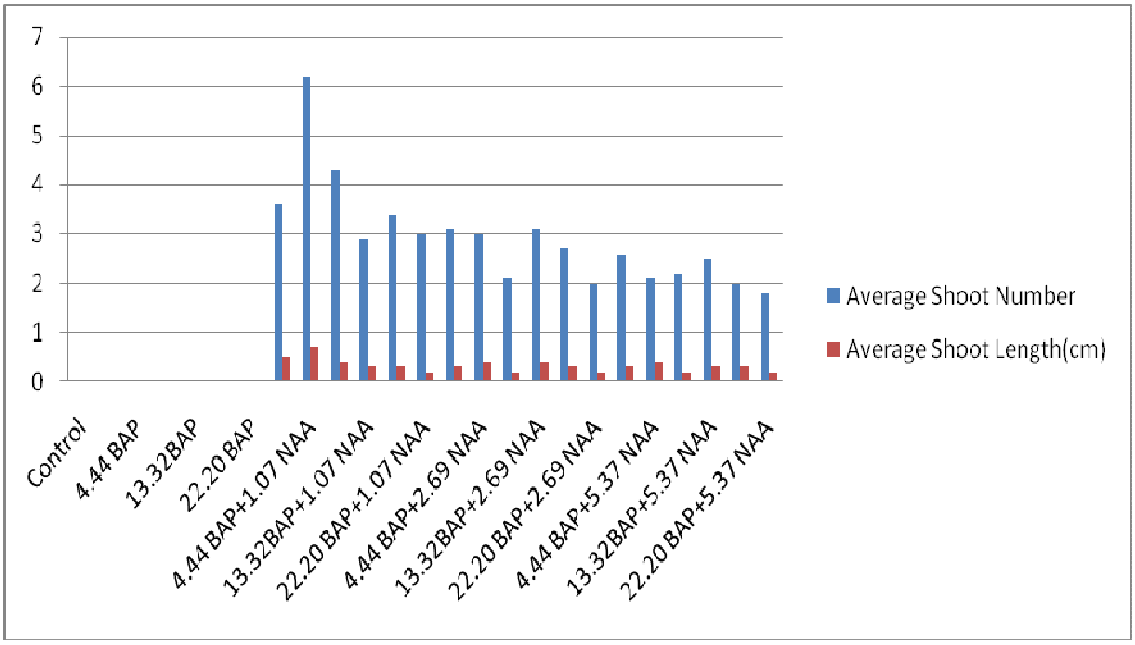
Experiments were done in triplicate and each treatment consisted of minimum ten replicates. The data was analysed using analysis of variance (ANOVA) of Completely randomized design (CRD) in GenStat Discovery Edition 13.The significance level was determined at P < = 0.05.Mean values of treatments were compared with Least Significance Difference (LSD).

Results:

***In vitro* shoot organogenesis**

De novo shoot organogenesis occurred only on proximal or middle sections of the roots. Distal regions of root explants proved to be incompetent for the purpose. Among the different treatments tried for shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) was found to be most optimal giving an average of 6.7 shoots per explant with mean shoot length of 0.7 cm without any growth of lateral roots within a period of 4 weeks [Figure 1]. MS medium without any plant growth regulator or augmented with BAP alone proved to be ineffective for shoot regeneration. On increasing the concentration of NAA to 2.69 µM and 5.37 µM , an enhanced induction of lateral roots was observed with a marked decrease in shoot bud induction from explants.(Graph 1).

Graph 1: Effect of PGR (µM) Treatment on Adventitious Shoot Regeneration from Root Explants



***In vitro* shoot multiplication**

The media combinations reported to be most optimal in our previous study on *S.chirata* (Pant *et al*., 2010) were MS medium supplemented with different concentrations of BAP, IAA and /or Ads for shoot multiplication and half-strength MS containing IBA for subsequent rooting. Similar combinations were tried in the present study and favourable results were obtained. On transferring *in vitro* grown shoots onto multiplication media, all media combinations tested were capable of inducing multiplication within 2-3 wks. Final observations in terms of mean number of shoots and shoot length were recorded after a period of 4 weeks and 8 weeks. All the treatments were significantly superior to the control except the treatments C4 and C6. In C4 (MS + 13.32 µM BAP) the average shoot number after 4 weeks and in C6 [MS+ 13.32 µM BAP+ 2.85 µM IAA] the average shoot length after 8 weeks was at par with the control. Amongst all, treatment C8 was found to be the best in all parameters. In this combination(MS + 4.44 µM BAP + 2.85 µM IAA + 271.45µM Ads) an average of 10.0 number of shoots with shoot length 2.0 cm after 4 weeks and mean shoot number 18.7 with shoot length 4.0 cm after 8 weeks was recorded [Table1] [Figure 3] .

Table 1: Effect of Plant Growth Regulators on *In vitro* Multiplication of Regenerated Microshoots

Treatment	MS medium +PGR(µM)			Average number of shoots		Average length of shoots(cm)	
	BAP	IAA	Ads	After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
C1	0.0	0.0	0.0	3.5	4	0.7	1.0
C2	4.44	0.0	0.0	4.4	7.4	1.6	2.3
C3	8.88	0.0	0.0	5.4	15	2.3	3.3
C4	13.32	0.0	0.0	5.3	9.1	1.2	1.7
C5	4.44	2.85	0.0	7.3	10.7	1.2	1.8
C6	8.88	2.85	0.0	6.4	9.8	1.1	1.6
C7	13.32	2.85	0.0	6.3	12.4	1.3	2.4
C8	**4.44**	**2.85**	**271.45**	**10.0**	**18.7**	**2.0**	**4.0**
C9	8.88	2.85	271.45	7.4	13.4	1.7	4.6
C10	13.32	2.85	271.45	8.2	11.5	1.9	2.7
Mean				6.4	11.2	1.5	2.5
LSD				1.8	2.8	0.4	1.8

After standardizing the media combination for shoot multiplication, *in vitro* multiplied shoots were subcultured on MS medium fortified with 4.44 µM BAP for maintenance of cultures.

***In vitro* rooting**

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama 1970). For the purpose, regenerated shoots were inoculated onto rooting media. It was observed that root primordia emerged from the shoot base within 10 to 14 days after shoot inoculation in all the treatments. Observations regarding mean root number and root length were recorded after 4 weeks and 8 weeks. All treatments were significantly superior to control. Optimal response was observed in the treatment R4(4.90 µM IBA) with average number of 21.0 roots per shoot after 4-weeks, 28.9 after 10-weeks and average root length 0.9 cm after 4-weeks & 1.6 cm after 10-weeks was observed in [Table 2] [Figure 4].

Table 2: Effect of Different Concentrations of IBA on Rooting of *In-vitro* Regenerated Shoots.

Treatment	1/2 strength MS medium +IBA (µM)	Average number of roots		Average length of roots(cm)	
		After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
R1	0.0	1.6	1.8	0.2	0.2
R2	0.98	6.2	12.1	0.6	1.1
R3	2.46	7.1	13.5	0.8	1.3
R4	**4.90**	**21.0**	**28.9**	**0.9**	**1.6**
R5	9.80	14.0	20.7	0.6	1.1
Mean		10.0	15.4	0.6	1.1
LSD		5.4	6.2	0.3	0.3

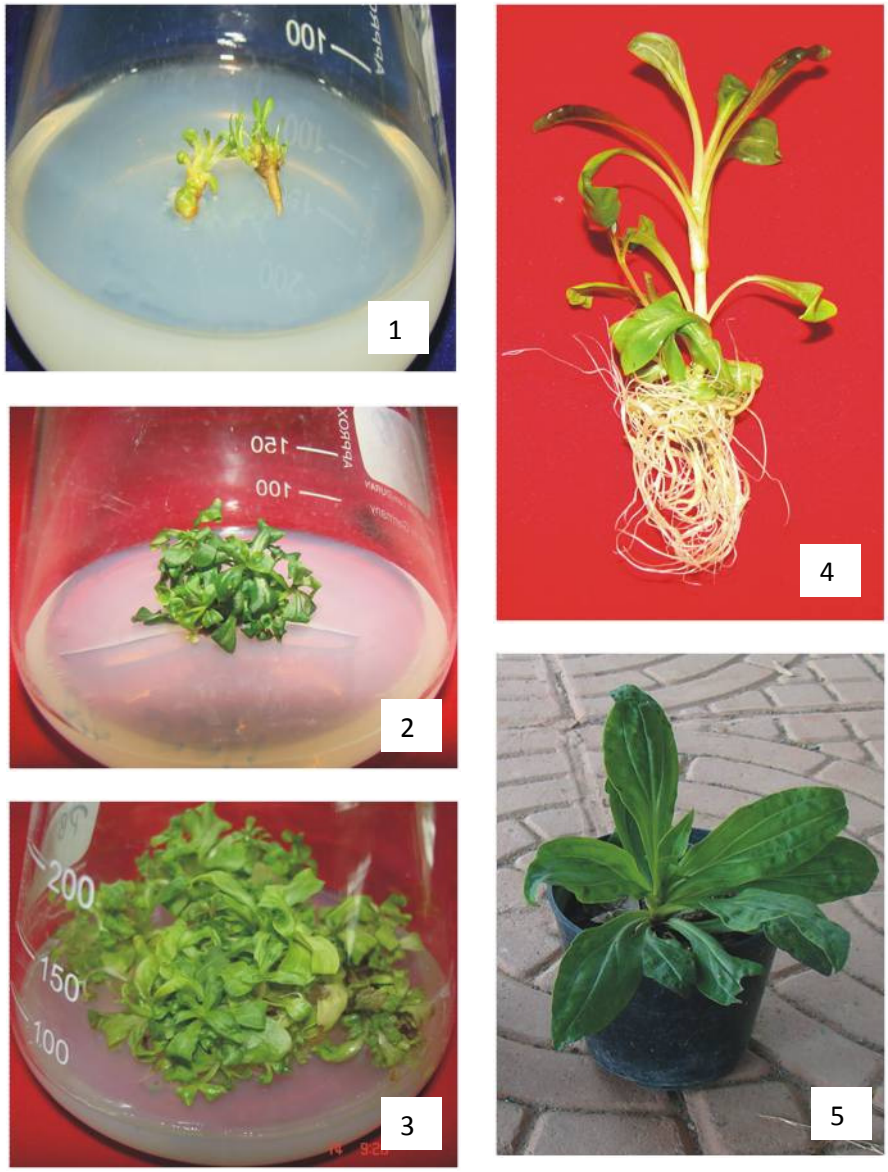


Fig. 1-5 Direct organogenesis of *Swertia chirata* via root explant:
(1) Shoot induction on root segments (2) Culture establishment
(3) Multiplication of shoots (4) *In vitro* rooted plantlet (5) Hardened plantlet.

Hardening and acclimatization

In-vitro hardening was done on liquid 1/4MS medium having 2% sucrose, for 7 days followed by acclimatization in agronet shade house in polybags containing a mixture of soil: sand :manure (1:1:1) and covered with perforated polythene bags. These polythene bags were withdrawn periodically and on emergence of new leaves, the polybags were completely removed. The plants maintained in shade house were irrigated with water. For next one month,

plants were maintained in net house. Well-acclimatized plants were shifted to pots containing soil and gave percentage survival of over 80%. [Figure 5].

Discussion

Careful observations revealed that of the different root segments tried for shoot bud initiation in our study, distal root segments remained non-responsive to shoot bud initiation while proximal and middle segments exhibited shoot regeneration and apparently no difference in the frequency and mode of shoot bud differentiation. Bud initiation and its formation uniformly from the proximal or middle portion of the root indicate that some secondary growth may also be essential for shoot initiation. The results are in corroboration with reports on other species where only the proximal and middle root sections exhibited *de novo* shoot organogenesis in *Comptonia peregrine* (Goforth and Torrey,1977), *Brassica napus* (Sharma and Thorpe, 1987), *Holostemma annulare* (Sudha *et al.*, 2000), *Populus tremula* (Vinocur *et al*.,2000), *Hypericum perforatum* (Zobayed and Saxena, 2003).

In the present study, among the different treatments tried for *in vitro* shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) gave maximum number of shoots without any growth of lateral roots within a period of 3 weeks . A synergistic effect of BAP and NAA has been highlighted in previous studies on shoot initiation from root explants of *Dalbergia sissoo* (Mukhopadhay and Mohan Ram,1981); *Citrus sinensis* (Burger and Hackett, 1986); *Citrus mitis* (Sim *et al.*, 1989); *Averrhoa carambola* (Kantharajah *et al.*,1992); *Clitoria ternatea* (Shahzad *et al.,*2007) and *Podophyllum hexandrum* (Chakraborty *et al*. ,2010) .

However, in our study, on increasing the concentration of NAA to 2.69 µM and 5.37 µM, significant reduction in shoot bud initiation was observed with an enhanced induction of lateral roots from cut end of roots. Root explants in our study were taken from *in vitro* regenerated roots growing in auxin-rich medium. On placing the explants in another auxin- rich medium may be an inhibiting factor for shoot bud differentiation while facilitating lateral root formation. This is also supported by the view that relatively high concentration of auxin suppresses the inception of shoot buds and promotes growth of lateral roots (Peterson, 1975). Cytokinins are considered to stimulate bud formation and inhibit lateral root formation. There are reports where BAP alone has found to be most efficient for shoot bud regeneration from root tissues in *Citrus mitis* (Sim *et al.,* 1989); *Citrus aurantifolia* (Bhat *et al.*, 1992); *Holostemma annulare* (Sudha *et al*., 2000); *Garnicia indica* (Deodhar *et al.*, 2000); *Blackstonia perfoliata* (Bijelovic *et al*. ,2004); *Crataeva nurvula* (Walia *et al.*,2004); *Melia azedarach* (Vila *et al.*, 2005). Contrastingly, in our study BAP when tried alone at different concentrations was found to be completely inefficient for shoot regeneration.

Wawrosch *et al.,* 1999 reported adventitious shoot regeneration from root explants of *S. chirata* where radicle explants from 3-week-old aseptically grown seedlings were used as primary explants for shoot organogenesis. They optimized a procedure of 3 weeks culturing root explants on BAP(3µM) supplemented MS medium followed by transfer to basal MS medium for another 3 weeks. However, the regeneration procedure described was not very efficient as only 1.9 shoots per explant could be achieved and the regenerated shoots were hyperhydrated. We report a significant improvement over these results as an average of 6.7 shoots per root explants could be obtained in our study which were further multiplied on MS medium supplemented with BAP, IAA and Ads and 18.0 fold multiplication was achieved. The regenerated shoots were healthy and robust without any observation of hyperhydration.

Rooting experiments were initiated after 180 days of subculturing to get maximum material for trying different hormone concentrations. Half-strength MS medium supplemented with IBA at 4.90 µM concentration proved to be most optimal for rooting. The results were similar to our previous findings on *in vitro* propagation of *S. chirata* via nodal explants. Wawrosch *et al.*(1999) had reported callus formation on exposure of shoots to media supplemented with upto 10 µM auxins for rooting. Therefore, they described a method of 2second dipping of shoots in an aqueous solution of 15 ppm NAA followed by 3-4 week cultivation period on hormone-free, half-strength MS medium for root development. In contrast, callusing did not occur in any of the treatments tried for *in vitro* rooting in our study and long, healthy root system developed on IBA augmented medium.

In vitro raised plants are heterotrophic in their mode of nutrition and cannot withstand the environmental conditions without proper hardening and acclimatization, thus, need to be hardened and acclimatized before field transplantation. Regenerated plantlets were hardened under controlled conditions of culture room on ¼ liquid MS medium and sucrose supply was decreased to 2%. After a period of one week, these plantlets were transferred to a rooting mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags in shade. Holes were made in polythene bags for gaseous

exchange. For acclimatizing the plantlets, bags were withdrawn periodically. During the process, the shoots elongated, leaves turned greener and expanded after which the polybags were completely removed. Hardened plants were subsequently transferred to soil filled in pots and over 80 % survivability was recorded.

We suggest that this ability of *S.chirata* plants of direct shoot organogenesis via root in nature, juvenile tissues of the root and specific combinations of growth regulators collectively triggered the process of direct shoot differentiation from root explants *in vitro*.

Conclusion

This study has revealed the morphogenetic potential of *in vitro* grown roots of *S.chirata* as a source tissue for micropropagation. The explants can be easily and regularly obtained from established shoot cultures and do not require disinfection treatment hence being ideal for germplasm exchange and cryopreservation. Normal root culture establishment for a number of plant species have the ability to accumulate secondary products similar to those found in mother donor plant (Tabata *et al.*, 1972; Whitten *et al.,* 1981). In *S.chirata* root being the most important part of the plant for medicinal purposes holds importance as the starting material for tissue culture studies. The present protocol will therefore serve as alternative means for *in vitro* clonal propagation of this endangered plant with immense medicinal value and further biochemical and physiological investigations.

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De novo Shoot Organogenesis from Cultured Root Explants of *Swertia chirata* Buch.-Ham.ex Wall.: An Endangered Medicinal Plant.

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Abstract: *In vitro* regeneration of plants from root cultures of *Swertia chirata* Buch.-Ham. ex Wall was obtained. Root explants from axenic shoot cultures were used for shoot induction. Optimal shoot regeneration without any callus intervention was observed on 1/2 MS (Murashige and Skoog's medium, 1962) + 4.44 µM 6, benzylaminopurine (BAP) +1.07 µM α-naphthalene acetic acid (NAA).Regenerated shoots were further multiplied on full strength MS medium supplemented with different concentrations of plant growth regulators. Maximum shoot multiplication was achieved on MS medium fortified with 4.44 µM BAP + 2.85 µM indole-3 acetic acid (IAA) + 271.45 µM adenine sulphate (Ads). Best results of rooting were observed on 1/2 strength MS medium containing 4.90 µM IBA. Plants with well-developed shoots and roots were successfully hardened with over 80% survivability.
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Keywords: *Swertia chirata*, root culture, *de novo* organogenesis, *in vitro* plant regeneration.

Abbreviations: MS: Murashige and Skoog (1962); **BAP:** 6, Benzylaminopurine; **Kn:** 6-Furfurylaminopurine; **NAA:** α-Naphthalene Acetic Acid; **IBA:** Indole-3 butyric acid; **IAA**: Indole-3 acetic acid; **GA_3**: Gibberellic acid; **Ads:** Adenine sulphate; **PGR:** Plant growth regulator

Introduction

Swertia chirata Buch.- Ham.- commonly known as chirayata is a medicinal plant of family Gentianaceae is native to temperate Himalaya (altitude above 4,000-10,000 ft.). The plant has an erect, about 2-3 ft long stem and the whole plant is bitter in taste. *S.chirata* contains a yellow bitter ophelic acid and two bitter glucosides chiratin and amarogentin. The root is said to be the most powerful part which is included in the British and American Pharmacopoeias and is used against a variety of diseases. The bitterness, antihelmintic, hypoglycemic and antipyretic properties are attributed to amarogentin ; most bitter compound isolated till date (Karan et al., 1996), swerchirin, swertiamarin and other active principles of the herb. The whole plant contains gentiamine alkaloids and the aerial part contains xanthones. Herbal medicines such as Ayush-64, Diabecon, Mensturyl syrup and Melicon ointment contain chirata extract in different amounts (Edwin et al., 1988, Valecha et al., 2000 and Mitra et al., 1996). The drug obtained from the dried plant is held in high esteem in India and is prescribed in a variety of forms and combinations in chronic fever, anemia and is used as special remedy for bronchial asthma, liver and stomach disorders, malaria and diabetes.

Several species of the genus *Swertia* are available in India and other countries and used as adulterant. However, *S.chirata* from India *and S.japonica* and *S.pseudochinenesis* from Japan are considered elite species because of their medicinal value and revenue-generating potential.

Chirayata is difficult to propagate on mass scale via seed owing to non-availability of seeds or due to harvesting of plants before seeds mature. The species is, therefore, deprived of natural regeneration. Also, the low viability and germination percentage of seeds and the necessary delicate field handling of the seedlings are some of the factors that discourage agro technology development and commercial cultivation of the species. There is an increasing pharmaceutical demand of the species both, in the indigenous and the world market but lack of large-scale commercial plantations of the herb continues. Destructive harvesting and loss of habitat has diminished the existing populations of *S.chirata*. The species is among the highly prioritized medicinal plants of India as identified by National Medicinal Plant Board, Govt. Of India and according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria, this priority plant has been designated as critically endangered .The need to develop techniques for its mass multiplication through different regeneration pathways thus, becomes imperative.

Documented literature reveals that there are limited reports on *in vitro* propagation of *Swertia chirata.* Micropropagation via field-grown nodal explants has been reported by Ahuja *et al.* (2003), Chaudhuri *et al.* (2007), Koul *et al*. (2009) and Pant *et al.,* 2010. Joshi and Dhawan (2007 a) and Balaraju *et al.* (2009) published reports on *in vitro* propagation of *S. chirata* using shoot tip explants derived from *in vitro* grown seedlings. ISSR marker analysis of genetic diversity among *S.chirata* genotypes of temperate Himalayan region of India was done by Joshi and Dhawan (2007 b). Chaudhuri *et al.* 2008 and 2009 reported direct shoot regeneration from *in vitro* leaves regeneration via immature seed cultures of *S. chirata*. Wang *et al.* (2009) described *in vitro* shoot regeneration from leaves taken from field-grown plants.

Root tissue has been proven to be highly regenerative explant and is relatively easy to maintain and manipulate *in vitro* (Vincour *et al.*, 2000; Franklin, 2004). Besides, shoots developing on root segments of the same plant have been reported to be genetically uniform and the method, an alternative way for clonal propagation. (Ostazeski and Henson, 1965; Budd, 1973; Chaturvedi *et al.*, 1981; Sharma *et al.*, 1993). The only available report on culture of root segments of *S.chirata* procured from *in vitro* raised seedlings was by Wawrosch *et al.* (1999). However, low frequency of shoot regeneration and hyperhydration of shoots hindered the production of quality shoots employing the procedure described by them. The present report communicates a reproducible protocol for regeneration of well-developed and healthy *S. chirata* plantlets via root segment culture without any intervening callus phase. The findings will be useful in conservation and mass multiplication of this endangered medicinal plant.

Material and Methods

Explant Source

In our previous study on *S.chirata* (Pant *et al.,* 2010) the shoots were induced from nodal explants. Regenerated shoots were further multiplied , subcultured and maintained on full strength MS medium supplemented with BAP(4.44 µM). Shoots having 1 or 2 nodes were rooted on half-strength MS medium supplemented with 4.90 µM indole-3 butyric acid (IBA).Ten – weeks old *in vitro* developed roots were used as an explant material for direct shoot organogenesis. For this, *in vitro* regenerated shoots via nodal explants were rooted on half-strength MS medium supplemented with 2% sucrose and IBA (4.90 µM). Aseptically removed long roots were repeatedly washed in sterile distilled water to free them from agar adhering to the surface. Subsequently they were dissected into 2-3 cm long proximal, middle and distal segments and placed horizontally on the culture medium for shoot organogenesis.

In vitro Shoot Organogenesis

Half-strength MS medium solidified with 0.7% agar (w/v) and supplemented with 2% sucrose and varying concentrations and combinations of phytohormones viz BAP(2.22 µM-22.20 µM) and NAA(1.07 µM- 5.37 µM) was tested for direct shoot regeneration from axenic root cultures. MS medium lacking plant growth regulators served as control. Data on average shoot number per explant and average shoot length (cm) was recorded after an interval of 4 weeks.

In vitro Shoot Multiplication

Once the culture conditions for optimum shoot induction from root explants were established, the proliferated shoots were subcultured onto fresh medium of same composition to establish an initial stock of shoots. These shoots were further used to assess the effect of growth regulators on further *in vitro* shoot multiplication. Full strength MS medium agarified with 0.7% agar (w/v) and fortified with 3% sucrose and varying concentrations and combinations of plant growth regulators were used for the purpose. Experiments conducted to obtain maximum shoot multiplication largely consisted of experiments pertaining to cytokinin (4.44 µM – 13.32 µM BAP) alone and in combination with auxin (1.14 µM – 2.85 µM IAA) and/or adjuvant adenine sulphate (271.45 µM). Observations pertaining to average shoot number and average shoot length were recorded after periodic intervals of 4 weeks and 8 weeks. Routine sub culturing of multiple shoots was carried out at an interval of every 3-week.

In vitro Rooting

Experiments on *in vitro* rooting of *in vitro* grown shoots were attempted with 2-3 cm long shoots produced during shoot multiplication. Shoots were cultured in each tube on MS medium containing 2% sucrose and supplemented with auxins IAA (1.14 µM - 11.40 µM), IBA (0.98 µM - 9.80 µM) and NAA (1.07 µM - 10.74 µM). Single shoots were cultured for initiation of roots and observations were recorded after 4 weeks and 8 weeks. Rooting response was recorded in terms of average number of roots produced and average root length.

Medium and Culture conditions

The pH of medium was adjusted to 5.8 before adding 0.7% agar (w/v) and sterilized by autoclaving at 15lbs (1.8 kg/cm^2) pressure at 121^{0}C for 15 minutes. Cultures were incubated at 25 ± 1^{0}C

for 16 hours in light (illuminated by 40 watt fluorescent tubes, 50µE m^{-2} s^{-1}) and for 8 hours in dark cycle irradiance by cool fluorescent tubes.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to liquid 1/4 strength MS strength medium having 2% sucrose and devoid of any plant growth regulator, for 7 days in flasks. Plantlets with well- developed roots were then removed from culture flasks and the roots gently washed under running tap water. They were then transferred to polybags containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags and maintained in high-density double check agronet open shade house. The plantlets were suitably irrigated with tap water .The polythene bags were periodically withdrawn to acclimatize the plantlets and when new leaves started emerging the polybags were completely removed. Hardened plantlets were subsequently transferred to soil in pots for further growth and development.

Statistical Analysis

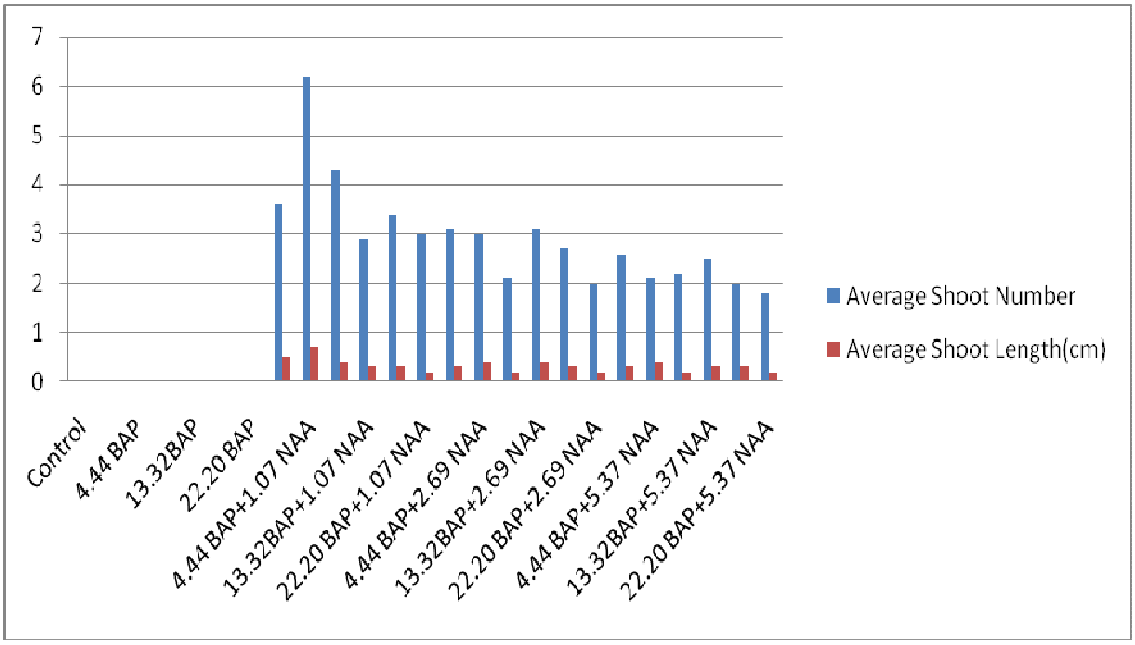
Experiments were done in triplicate and each treatment consisted of minimum ten replicates. The data was analysed using analysis of variance (ANOVA) of Completely randomized design (CRD) in GenStat Discovery Edition 13.The significance level was determined at P < = 0.05.Mean values of treatments were compared with Least Significance Difference (LSD).

Results:

***In vitro* shoot organogenesis**

De novo shoot organogenesis occurred only on proximal or middle sections of the roots. Distal regions of root explants proved to be incompetent for the purpose. Among the different treatments tried for shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) was found to be most optimal giving an average of 6.7 shoots per explant with mean shoot length of 0.7 cm without any growth of lateral roots within a period of 4 weeks [Figure 1]. MS medium without any plant growth regulator or augmented with BAP alone proved to be ineffective for shoot regeneration. On increasing the concentration of NAA to 2.69 µM and 5.37 µM , an enhanced induction of lateral roots was observed with a marked decrease in shoot bud induction from explants.(Graph 1).

Graph 1: Effect of PGR (µM) Treatment on Adventitious Shoot Regeneration from Root Explants



***In vitro* shoot multiplication**

The media combinations reported to be most optimal in our previous study on *S.chirata* (Pant *et al*., 2010) were MS medium supplemented with different concentrations of BAP, IAA and /or Ads for shoot multiplication and half-strength MS containing IBA for subsequent rooting. Similar combinations were tried in the present study and favourable results were obtained. On transferring *in vitro* grown shoots onto multiplication media, all media combinations tested were capable of inducing multiplication within 2-3 wks. Final observations in terms of mean number of shoots and shoot length were recorded after a period of 4 weeks and 8 weeks. All the treatments were significantly superior to the control except the treatments C4 and C6. In C4 (MS + 13.32 µM BAP) the average shoot number after 4 weeks and in C6 [MS+ 13.32 µM BAP+ 2.85 µM IAA] the average shoot length after 8 weeks was at par with the control. Amongst all, treatment C8 was found to be the best in all parameters. In this combination(MS + 4.44 µM BAP + 2.85 µM IAA + 271.45µM Ads) an average of 10.0 number of shoots with shoot length 2.0 cm after 4 weeks and mean shoot number 18.7 with shoot length 4.0 cm after 8 weeks was recorded [Table1] [Figure 3] .

Table 1: Effect of Plant Growth Regulators on *In vitro* Multiplication of Regenerated Microshoots

Treatment	MS medium +PGR(µM)			Average number of shoots		Average length of shoots(cm)	
	BAP	IAA	Ads	After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
C1	0.0	0.0	0.0	3.5	4	0.7	1.0
C2	4.44	0.0	0.0	4.4	7.4	1.6	2.3
C3	8.88	0.0	0.0	5.4	15	2.3	3.3
C4	13.32	0.0	0.0	5.3	9.1	1.2	1.7
C5	4.44	2.85	0.0	7.3	10.7	1.2	1.8
C6	8.88	2.85	0.0	6.4	9.8	1.1	1.6
C7	13.32	2.85	0.0	6.3	12.4	1.3	2.4
C8	**4.44**	**2.85**	**271.45**	**10.0**	**18.7**	**2.0**	**4.0**
C9	8.88	2.85	271.45	7.4	13.4	1.7	4.6
C10	13.32	2.85	271.45	8.2	11.5	1.9	2.7
Mean				6.4	11.2	1.5	2.5
LSD				1.8	2.8	0.4	1.8

After standardizing the media combination for shoot multiplication, *in vitro* multiplied shoots were subcultured on MS medium fortified with 4.44 µM BAP for maintenance of cultures.

***In vitro* rooting**

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama 1970). For the purpose, regenerated shoots were inoculated onto rooting media. It was observed that root primordia emerged from the shoot base within 10 to 14 days after shoot inoculation in all the treatments. Observations regarding mean root number and root length were recorded after 4 weeks and 8 weeks. All treatments were significantly superior to control. Optimal response was observed in the treatment R4(4.90 µM IBA) with average number of 21.0 roots per shoot after 4-weeks, 28.9 after 10-weeks and average root length 0.9 cm after 4-weeks & 1.6 cm after 10-weeks was observed in [Table 2] [Figure 4].

Table 2: Effect of Different Concentrations of IBA on Rooting of *In-vitro* Regenerated Shoots.

Treatment	1/2 strength MS medium +IBA (µM)	Average number of roots		Average length of roots(cm)	
		After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
R1	0.0	1.6	1.8	0.2	0.2
R2	0.98	6.2	12.1	0.6	1.1
R3	2.46	7.1	13.5	0.8	1.3
R4	**4.90**	**21.0**	**28.9**	**0.9**	**1.6**
R5	9.80	14.0	20.7	0.6	1.1
Mean		10.0	15.4	0.6	1.1
LSD		5.4	6.2	0.3	0.3

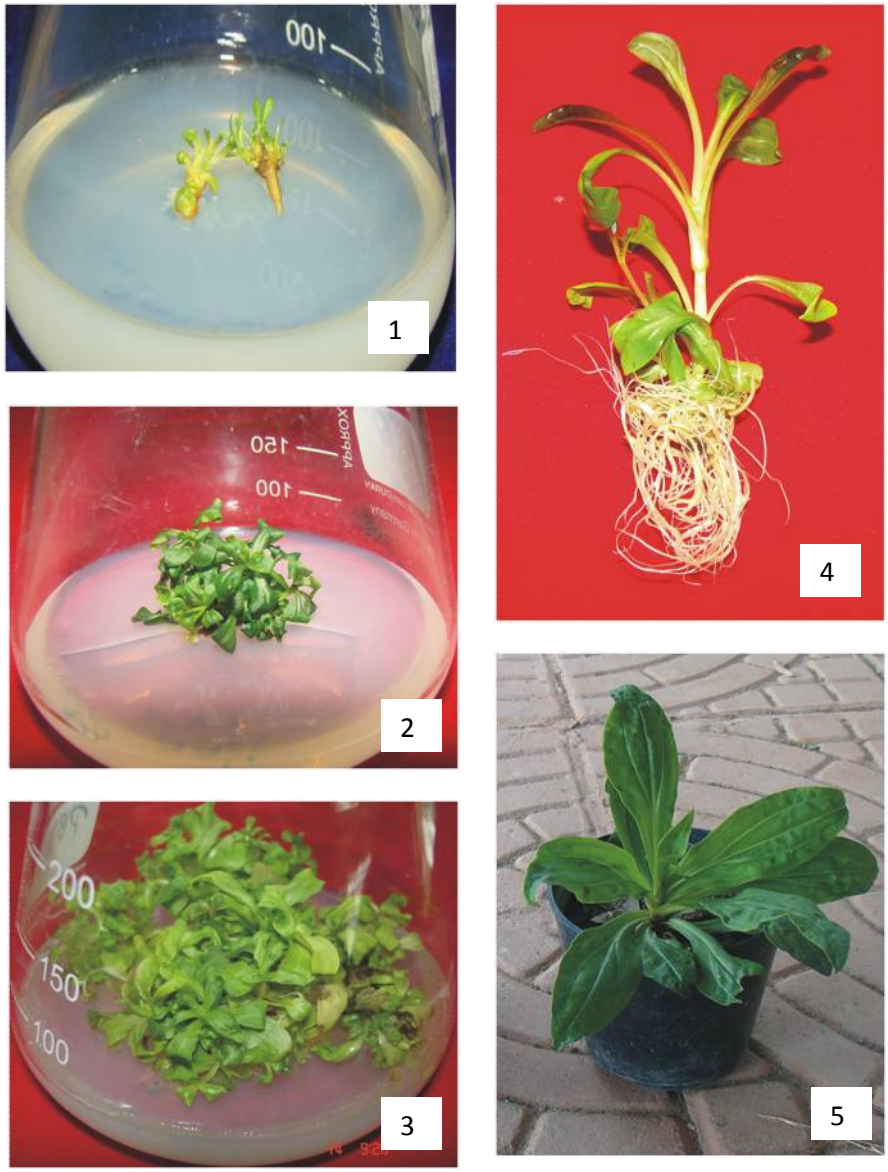


Fig. 1-5 Direct organogenesis of *Swertia chirata* via root explant:
(1) Shoot induction on root segments (2) Culture establishment
(3) Multiplication of shoots (4) *In vitro* rooted plantlet (5) Hardened plantlet.

Hardening and acclimatization

In-vitro hardening was done on liquid 1/4MS medium having 2% sucrose, for 7 days followed by acclimatization in agronet shade house in polybags containing a mixture of soil: sand :manure (1:1:1) and covered with perforated polythene bags. These polythene bags were withdrawn periodically and on emergence of new leaves, the polybags were completely removed. The plants maintained in shade house were irrigated with water. For next one month,

plants were maintained in net house. Well-acclimatized plants were shifted to pots containing soil and gave percentage survival of over 80%. [Figure 5].

Discussion

Careful observations revealed that of the different root segments tried for shoot bud initiation in our study, distal root segments remained non-responsive to shoot bud initiation while proximal and middle segments exhibited shoot regeneration and apparently no difference in the frequency and mode of shoot bud differentiation. Bud initiation and its formation uniformly from the proximal or middle portion of the root indicate that some secondary growth may also be essential for shoot initiation. The results are in corroboration with reports on other species where only the proximal and middle root sections exhibited *de novo* shoot organogenesis in *Comptonia peregrine* (Goforth and Torrey,1977), *Brassica napus* (Sharma and Thorpe, 1987), *Holostemma annulare* (Sudha *et al.*, 2000), *Populus tremula* (Vinocur *et al*.,2000), *Hypericum perforatum* (Zobayed and Saxena, 2003).

In the present study, among the different treatments tried for *in vitro* shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) gave maximum number of shoots without any growth of lateral roots within a period of 3 weeks . A synergistic effect of BAP and NAA has been highlighted in previous studies on shoot initiation from root explants of *Dalbergia sissoo* (Mukhopadhay and Mohan Ram,1981); *Citrus sinensis* (Burger and Hackett, 1986); *Citrus mitis* (Sim *et al.*, 1989); *Averrhoa carambola* (Kantharajah *et al.*,1992); *Clitoria ternatea* (Shahzad *et al.,*2007) and *Podophyllum hexandrum* (Chakraborty *et al*. ,2010) .

However, in our study, on increasing the concentration of NAA to 2.69 µM and 5.37 µM, significant reduction in shoot bud initiation was observed with an enhanced induction of lateral roots from cut end of roots. Root explants in our study were taken from *in vitro* regenerated roots growing in auxin-rich medium. On placing the explants in another auxin- rich medium may be an inhibiting factor for shoot bud differentiation while facilitating lateral root formation. This is also supported by the view that relatively high concentration of auxin suppresses the inception of shoot buds and promotes growth of lateral roots (Peterson, 1975). Cytokinins are considered to stimulate bud formation and inhibit lateral root formation. There are reports where BAP alone has found to be most efficient for shoot bud regeneration from root tissues in *Citrus mitis* (Sim *et al.,* 1989); *Citrus aurantifolia* (Bhat *et al.*, 1992); *Holostemma annulare* (Sudha *et al*., 2000); *Garnicia indica* (Deodhar *et al.*, 2000); *Blackstonia perfoliata* (Bijelovic *et al*. ,2004); *Crataeva nurvula* (Walia *et al.*,2004); *Melia azedarach* (Vila *et al.*, 2005). Contrastingly, in our study BAP when tried alone at different concentrations was found to be completely inefficient for shoot regeneration.

Wawrosch *et al.,* 1999 reported adventitious shoot regeneration from root explants of *S. chirata* where radicle explants from 3-week-old aseptically grown seedlings were used as primary explants for shoot organogenesis. They optimized a procedure of 3 weeks culturing root explants on BAP(3µM) supplemented MS medium followed by transfer to basal MS medium for another 3 weeks. However, the regeneration procedure described was not very efficient as only 1.9 shoots per explant could be achieved and the regenerated shoots were hyperhydrated. We report a significant improvement over these results as an average of 6.7 shoots per root explants could be obtained in our study which were further multiplied on MS medium supplemented with BAP, IAA and Ads and 18.0 fold multiplication was achieved. The regenerated shoots were healthy and robust without any observation of hyperhydration.

Rooting experiments were initiated after 180 days of subculturing to get maximum material for trying different hormone concentrations. Half-strength MS medium supplemented with IBA at 4.90 µM concentration proved to be most optimal for rooting. The results were similar to our previous findings on *in vitro* propagation of *S. chirata* via nodal explants. Wawrosch *et al.*(1999) had reported callus formation on exposure of shoots to media supplemented with upto 10 µM auxins for rooting. Therefore, they described a method of 2second dipping of shoots in an aqueous solution of 15 ppm NAA followed by 3-4 week cultivation period on hormone-free, half-strength MS medium for root development. In contrast, callusing did not occur in any of the treatments tried for *in vitro* rooting in our study and long, healthy root system developed on IBA augmented medium.

In vitro raised plants are heterotrophic in their mode of nutrition and cannot withstand the environmental conditions without proper hardening and acclimatization, thus, need to be hardened and acclimatized before field transplantation. Regenerated plantlets were hardened under controlled conditions of culture room on ¼ liquid MS medium and sucrose supply was decreased to 2%. After a period of one week, these plantlets were transferred to a rooting mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags in shade. Holes were made in polythene bags for gaseous

exchange. For acclimatizing the plantlets, bags were withdrawn periodically. During the process, the shoots elongated, leaves turned greener and expanded after which the polybags were completely removed. Hardened plants were subsequently transferred to soil filled in pots and over 80 % survivability was recorded.

We suggest that this ability of *S.chirata* plants of direct shoot organogenesis via root in nature, juvenile tissues of the root and specific combinations of growth regulators collectively triggered the process of direct shoot differentiation from root explants *in vitro*.

Conclusion

This study has revealed the morphogenetic potential of *in vitro* grown roots of *S.chirata* as a source tissue for micropropagation. The explants can be easily and regularly obtained from established shoot cultures and do not require disinfection treatment hence being ideal for germplasm exchange and cryopreservation. Normal root culture establishment for a number of plant species have the ability to accumulate secondary products similar to those found in mother donor plant (Tabata *et al.*, 1972; Whitten *et al.,* 1981). In *S.chirata* root being the most important part of the plant for medicinal purposes holds importance as the starting material for tissue culture studies. The present protocol will therefore serve as alternative means for *in vitro* clonal propagation of this endangered plant with immense medicinal value and further biochemical and physiological investigations.

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De novo Shoot Organogenesis from Cultured Root Explants of *Swertia chirata* Buch.-Ham.ex Wall.: An Endangered Medicinal Plant.

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Abstract: *In vitro* regeneration of plants from root cultures of *Swertia chirata* Buch.-Ham. ex Wall was obtained. Root explants from axenic shoot cultures were used for shoot induction. Optimal shoot regeneration without any callus intervention was observed on 1/2 MS (Murashige and Skoog's medium, 1962) + 4.44 µM 6, benzylaminopurine (BAP) +1.07 µM α-naphthalene acetic acid (NAA).Regenerated shoots were further multiplied on full strength MS medium supplemented with different concentrations of plant growth regulators. Maximum shoot multiplication was achieved on MS medium fortified with 4.44 µM BAP + 2.85 µM indole-3 acetic acid (IAA) + 271.45 µM adenine sulphate (Ads). Best results of rooting were observed on 1/2 strength MS medium containing 4.90 µM IBA. Plants with well-developed shoots and roots were successfully hardened with over 80% survivability.
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Keywords: *Swertia chirata*, root culture, *de novo* organogenesis, *in vitro* plant regeneration.

Abbreviations: MS: Murashige and Skoog (1962); **BAP:** 6, Benzylaminopurine; **Kn:** 6-Furfurylaminopurine; **NAA:** α-Naphthalene Acetic Acid; **IBA:** Indole-3 butyric acid; **IAA**: Indole-3 acetic acid; **GA_3**: Gibberellic acid; **Ads:** Adenine sulphate; **PGR:** Plant growth regulator

Introduction

Swertia chirata Buch.- Ham.- commonly known as chirayata is a medicinal plant of family Gentianaceae is native to temperate Himalaya (altitude above 4,000-10,000 ft.). The plant has an erect, about 2-3 ft long stem and the whole plant is bitter in taste. *S.chirata* contains a yellow bitter ophelic acid and two bitter glucosides chiratin and amarogentin. The root is said to be the most powerful part which is included in the British and American Pharmacopoeias and is used against a variety of diseases. The bitterness, antihelmintic, hypoglycemic and antipyretic properties are attributed to amarogentin ; most bitter compound isolated till date (Karan et al., 1996), swerchirin, swertiamarin and other active principles of the herb. The whole plant contains gentiamine alkaloids and the aerial part contains xanthones. Herbal medicines such as Ayush-64, Diabecon, Mensturyl syrup and Melicon ointment contain chirata extract in different amounts (Edwin et al., 1988, Valecha et al., 2000 and Mitra et al., 1996). The drug obtained from the dried plant is held in high esteem in India and is prescribed in a variety of forms and combinations in chronic fever, anemia and is used as special remedy for bronchial asthma, liver and stomach disorders, malaria and diabetes.

Several species of the genus *Swertia* are available in India and other countries and used as adulterant. However, *S.chirata* from India *and S.japonica* and *S.pseudochinenesis* from Japan are considered elite species because of their medicinal value and revenue-generating potential.

Chirayata is difficult to propagate on mass scale via seed owing to non-availability of seeds or due to harvesting of plants before seeds mature. The species is, therefore, deprived of natural regeneration. Also, the low viability and germination percentage of seeds and the necessary delicate field handling of the seedlings are some of the factors that discourage agro technology development and commercial cultivation of the species. There is an increasing pharmaceutical demand of the species both, in the indigenous and the world market but lack of large-scale commercial plantations of the herb continues. Destructive harvesting and loss of habitat has diminished the existing populations of *S.chirata*. The species is among the highly prioritized medicinal plants of India as identified by National Medicinal Plant Board, Govt. Of India and according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria, this priority plant has been designated as critically endangered .The need to develop techniques for its mass multiplication through different regeneration pathways thus, becomes imperative.

Documented literature reveals that there are limited reports on *in vitro* propagation of *Swertia chirata.* Micropropagation via field-grown nodal explants has been reported by Ahuja *et al.* (2003), Chaudhuri *et al.* (2007), Koul *et al*. (2009) and Pant *et al.,* 2010. Joshi and Dhawan (2007 a) and Balaraju *et al.* (2009) published reports on *in vitro* propagation of *S. chirata* using shoot tip explants derived from *in vitro* grown seedlings. ISSR marker analysis of genetic diversity among *S.chirata* genotypes of temperate Himalayan region of India was done by Joshi and Dhawan (2007 b). Chaudhuri *et al.* 2008 and 2009 reported direct shoot regeneration from *in vitro* leaves regeneration via immature seed cultures of *S. chirata*. Wang *et al.* (2009) described *in vitro* shoot regeneration from leaves taken from field-grown plants.

Root tissue has been proven to be highly regenerative explant and is relatively easy to maintain and manipulate *in vitro* (Vincour *et al.*, 2000; Franklin, 2004). Besides, shoots developing on root segments of the same plant have been reported to be genetically uniform and the method, an alternative way for clonal propagation. (Ostazeski and Henson, 1965; Budd, 1973; Chaturvedi *et al.*, 1981; Sharma *et al.*, 1993). The only available report on culture of root segments of *S.chirata* procured from *in vitro* raised seedlings was by Wawrosch *et al.* (1999). However, low frequency of shoot regeneration and hyperhydration of shoots hindered the production of quality shoots employing the procedure described by them. The present report communicates a reproducible protocol for regeneration of well-developed and healthy *S. chirata* plantlets via root segment culture without any intervening callus phase. The findings will be useful in conservation and mass multiplication of this endangered medicinal plant.

Material and Methods

Explant Source

In our previous study on *S.chirata* (Pant *et al.,* 2010) the shoots were induced from nodal explants. Regenerated shoots were further multiplied , subcultured and maintained on full strength MS medium supplemented with BAP(4.44 µM). Shoots having 1 or 2 nodes were rooted on half-strength MS medium supplemented with 4.90 µM indole-3 butyric acid (IBA).Ten – weeks old *in vitro* developed roots were used as an explant material for direct shoot organogenesis. For this, *in vitro* regenerated shoots via nodal explants were rooted on half-strength MS medium supplemented with 2% sucrose and IBA (4.90 µM). Aseptically removed long roots were repeatedly washed in sterile distilled water to free them from agar adhering to the surface. Subsequently they were dissected into 2-3 cm long proximal, middle and distal segments and placed horizontally on the culture medium for shoot organogenesis.

In vitro Shoot Organogenesis

Half-strength MS medium solidified with 0.7% agar (w/v) and supplemented with 2% sucrose and varying concentrations and combinations of phytohormones viz BAP(2.22 µM-22.20 µM) and NAA(1.07 µM- 5.37 µM) was tested for direct shoot regeneration from axenic root cultures. MS medium lacking plant growth regulators served as control. Data on average shoot number per explant and average shoot length (cm) was recorded after an interval of 4 weeks.

In vitro Shoot Multiplication

Once the culture conditions for optimum shoot induction from root explants were established, the proliferated shoots were subcultured onto fresh medium of same composition to establish an initial stock of shoots. These shoots were further used to assess the effect of growth regulators on further *in vitro* shoot multiplication. Full strength MS medium agarified with 0.7% agar (w/v) and fortified with 3% sucrose and varying concentrations and combinations of plant growth regulators were used for the purpose. Experiments conducted to obtain maximum shoot multiplication largely consisted of experiments pertaining to cytokinin (4.44 µM – 13.32 µM BAP) alone and in combination with auxin (1.14 µM – 2.85 µM IAA) and/or adjuvant adenine sulphate (271.45 µM). Observations pertaining to average shoot number and average shoot length were recorded after periodic intervals of 4 weeks and 8 weeks. Routine sub culturing of multiple shoots was carried out at an interval of every 3-week.

In vitro Rooting

Experiments on *in vitro* rooting of *in vitro* grown shoots were attempted with 2-3 cm long shoots produced during shoot multiplication. Shoots were cultured in each tube on MS medium containing 2% sucrose and supplemented with auxins IAA (1.14 µM - 11.40 µM), IBA (0.98 µM - 9.80 µM) and NAA (1.07 µM - 10.74 µM). Single shoots were cultured for initiation of roots and observations were recorded after 4 weeks and 8 weeks. Rooting response was recorded in terms of average number of roots produced and average root length.

Medium and Culture conditions

The pH of medium was adjusted to 5.8 before adding 0.7% agar (w/v) and sterilized by autoclaving at 15lbs (1.8 kg/cm^2) pressure at 121^{0}C for 15 minutes. Cultures were incubated at 25 ± 1^{0}C

for 16 hours in light (illuminated by 40 watt fluorescent tubes, 50µE m^{-2} s^{-1}) and for 8 hours in dark cycle irradiance by cool fluorescent tubes.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to liquid 1/4 strength MS strength medium having 2% sucrose and devoid of any plant growth regulator, for 7 days in flasks. Plantlets with well- developed roots were then removed from culture flasks and the roots gently washed under running tap water. They were then transferred to polybags containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags and maintained in high-density double check agronet open shade house. The plantlets were suitably irrigated with tap water .The polythene bags were periodically withdrawn to acclimatize the plantlets and when new leaves started emerging the polybags were completely removed. Hardened plantlets were subsequently transferred to soil in pots for further growth and development.

Statistical Analysis

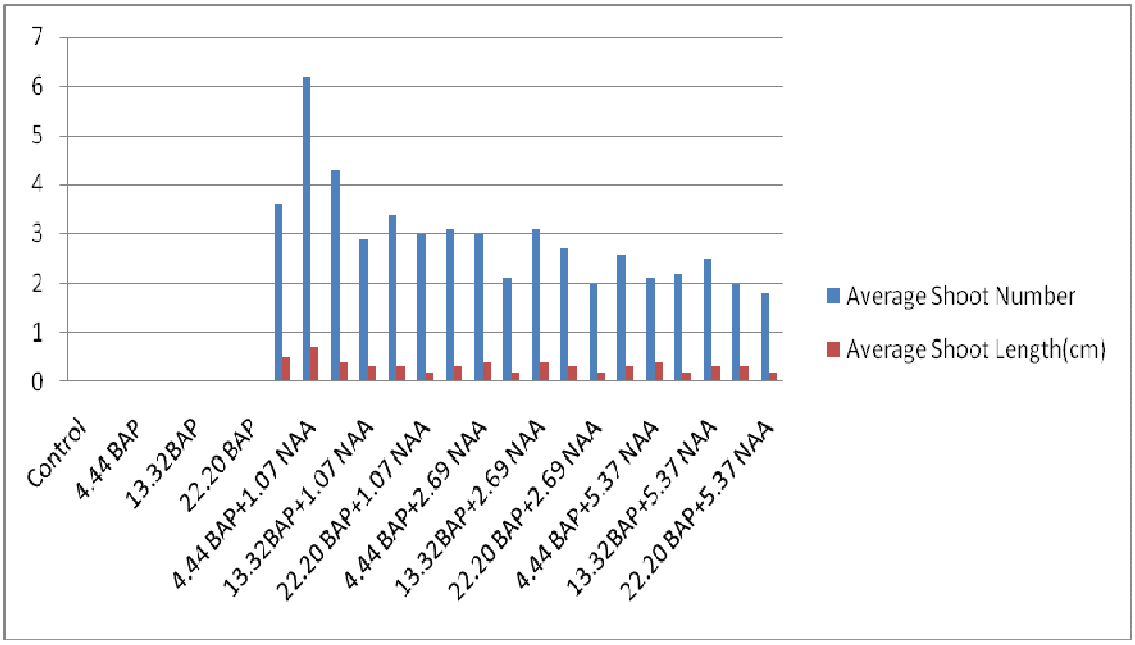
Experiments were done in triplicate and each treatment consisted of minimum ten replicates. The data was analysed using analysis of variance (ANOVA) of Completely randomized design (CRD) in GenStat Discovery Edition 13.The significance level was determined at P < = 0.05.Mean values of treatments were compared with Least Significance Difference (LSD).

Results:

***In vitro* shoot organogenesis**

De novo shoot organogenesis occurred only on proximal or middle sections of the roots. Distal regions of root explants proved to be incompetent for the purpose. Among the different treatments tried for shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) was found to be most optimal giving an average of 6.7 shoots per explant with mean shoot length of 0.7 cm without any growth of lateral roots within a period of 4 weeks [Figure 1]. MS medium without any plant growth regulator or augmented with BAP alone proved to be ineffective for shoot regeneration. On increasing the concentration of NAA to 2.69 µM and 5.37 µM , an enhanced induction of lateral roots was observed with a marked decrease in shoot bud induction from explants.(Graph 1).

Graph 1: Effect of PGR (µM) Treatment on Adventitious Shoot Regeneration from Root Explants



***In vitro* shoot multiplication**

The media combinations reported to be most optimal in our previous study on *S.chirata* (Pant *et al*., 2010) were MS medium supplemented with different concentrations of BAP, IAA and /or Ads for shoot multiplication and half-strength MS containing IBA for subsequent rooting. Similar combinations were tried in the present study and favourable results were obtained. On transferring *in vitro* grown shoots onto multiplication media, all media combinations tested were capable of inducing multiplication within 2-3 wks. Final observations in terms of mean number of shoots and shoot length were recorded after a period of 4 weeks and 8 weeks. All the treatments were significantly superior to the control except the treatments C4 and C6. In C4 (MS + 13.32 µM BAP) the average shoot number after 4 weeks and in C6 [MS+ 13.32 µM BAP+ 2.85 µM IAA] the average shoot length after 8 weeks was at par with the control. Amongst all, treatment C8 was found to be the best in all parameters. In this combination(MS + 4.44 µM BAP + 2.85 µM IAA + 271.45µM Ads) an average of 10.0 number of shoots with shoot length 2.0 cm after 4 weeks and mean shoot number 18.7 with shoot length 4.0 cm after 8 weeks was recorded [Table1] [Figure 3] .

Table 1: Effect of Plant Growth Regulators on *In vitro* Multiplication of Regenerated Microshoots

Treatment	MS medium +PGR(µM)			Average number of shoots		Average length of shoots(cm)	
	BAP	IAA	Ads	After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
C1	0.0	0.0	0.0	3.5	4	0.7	1.0
C2	4.44	0.0	0.0	4.4	7.4	1.6	2.3
C3	8.88	0.0	0.0	5.4	15	2.3	3.3
C4	13.32	0.0	0.0	5.3	9.1	1.2	1.7
C5	4.44	2.85	0.0	7.3	10.7	1.2	1.8
C6	8.88	2.85	0.0	6.4	9.8	1.1	1.6
C7	13.32	2.85	0.0	6.3	12.4	1.3	2.4
C8	**4.44**	**2.85**	**271.45**	**10.0**	**18.7**	**2.0**	**4.0**
C9	8.88	2.85	271.45	7.4	13.4	1.7	4.6
C10	13.32	2.85	271.45	8.2	11.5	1.9	2.7
Mean				6.4	11.2	1.5	2.5
LSD				1.8	2.8	0.4	1.8

After standardizing the media combination for shoot multiplication, *in vitro* multiplied shoots were subcultured on MS medium fortified with 4.44 µM BAP for maintenance of cultures.

***In vitro* rooting**

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama 1970). For the purpose, regenerated shoots were inoculated onto rooting media. It was observed that root primordia emerged from the shoot base within 10 to 14 days after shoot inoculation in all the treatments. Observations regarding mean root number and root length were recorded after 4 weeks and 8 weeks. All treatments were significantly superior to control. Optimal response was observed in the treatment R4(4.90 µM IBA) with average number of 21.0 roots per shoot after 4-weeks, 28.9 after 10-weeks and average root length 0.9 cm after 4-weeks & 1.6 cm after 10-weeks was observed in [Table 2] [Figure 4].

Table 2: Effect of Different Concentrations of IBA on Rooting of *In-vitro* Regenerated Shoots.

Treatment	1/2 strength MS medium +IBA (µM)	Average number of roots		Average length of roots(cm)	
		After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
R1	0.0	1.6	1.8	0.2	0.2
R2	0.98	6.2	12.1	0.6	1.1
R3	2.46	7.1	13.5	0.8	1.3
R4	**4.90**	**21.0**	**28.9**	**0.9**	**1.6**
R5	9.80	14.0	20.7	0.6	1.1
Mean		10.0	15.4	0.6	1.1
LSD		5.4	6.2	0.3	0.3

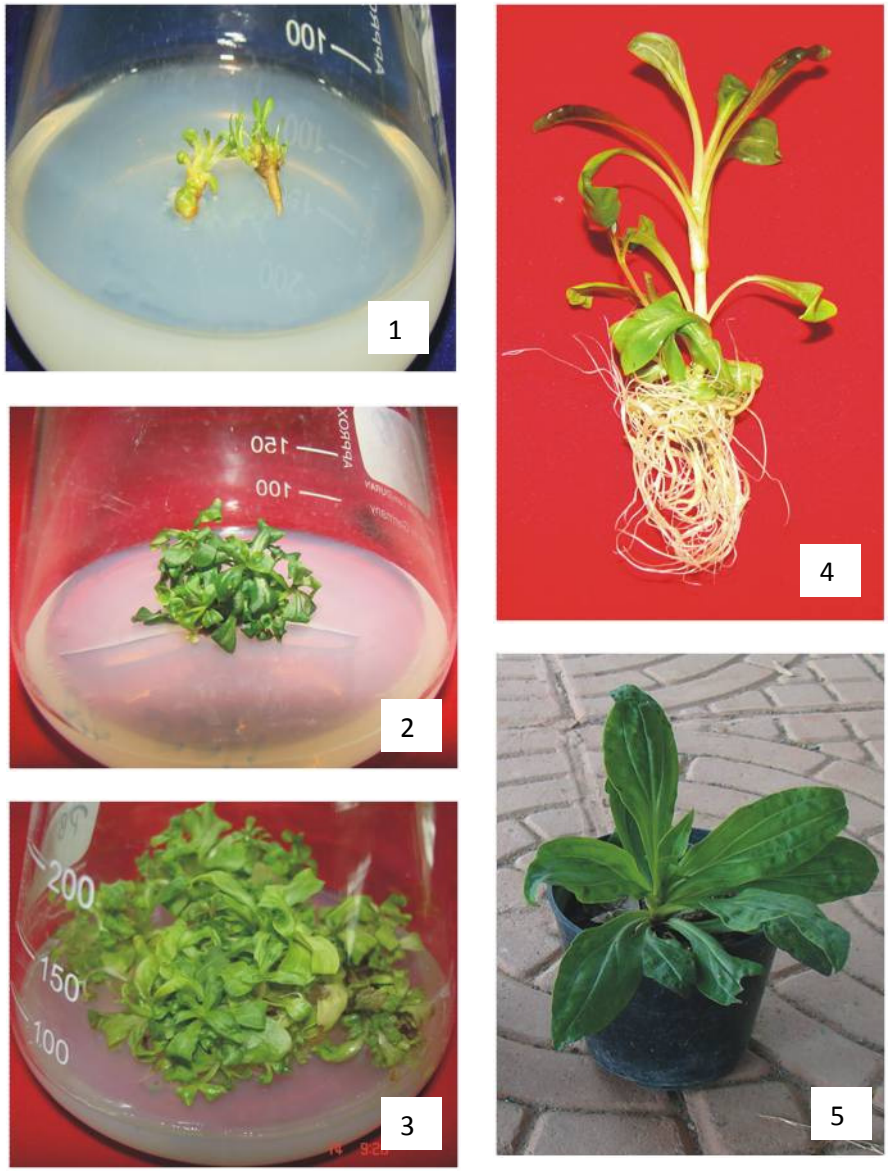


Fig. 1-5 Direct organogenesis of *Swertia chirata* via root explant:
(1) Shoot induction on root segments (2) Culture establishment
(3) Multiplication of shoots (4) *In vitro* rooted plantlet (5) Hardened plantlet.

Hardening and acclimatization

In-vitro hardening was done on liquid 1/4MS medium having 2% sucrose, for 7 days followed by acclimatization in agronet shade house in polybags containing a mixture of soil: sand :manure (1:1:1) and covered with perforated polythene bags. These polythene bags were withdrawn periodically and on emergence of new leaves, the polybags were completely removed. The plants maintained in shade house were irrigated with water. For next one month,

plants were maintained in net house. Well-acclimatized plants were shifted to pots containing soil and gave percentage survival of over 80%. [Figure 5].

Discussion

Careful observations revealed that of the different root segments tried for shoot bud initiation in our study, distal root segments remained non-responsive to shoot bud initiation while proximal and middle segments exhibited shoot regeneration and apparently no difference in the frequency and mode of shoot bud differentiation. Bud initiation and its formation uniformly from the proximal or middle portion of the root indicate that some secondary growth may also be essential for shoot initiation. The results are in corroboration with reports on other species where only the proximal and middle root sections exhibited *de novo* shoot organogenesis in *Comptonia peregrine* (Goforth and Torrey,1977), *Brassica napus* (Sharma and Thorpe, 1987), *Holostemma annulare* (Sudha *et al.*, 2000), *Populus tremula* (Vinocur *et al*.,2000), *Hypericum perforatum* (Zobayed and Saxena, 2003).

In the present study, among the different treatments tried for *in vitro* shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) gave maximum number of shoots without any growth of lateral roots within a period of 3 weeks . A synergistic effect of BAP and NAA has been highlighted in previous studies on shoot initiation from root explants of *Dalbergia sissoo* (Mukhopadhay and Mohan Ram,1981); *Citrus sinensis* (Burger and Hackett, 1986); *Citrus mitis* (Sim *et al.*, 1989); *Averrhoa carambola* (Kantharajah *et al.*,1992); *Clitoria ternatea* (Shahzad *et al.,*2007) and *Podophyllum hexandrum* (Chakraborty *et al*. ,2010) .

However, in our study, on increasing the concentration of NAA to 2.69 µM and 5.37 µM, significant reduction in shoot bud initiation was observed with an enhanced induction of lateral roots from cut end of roots. Root explants in our study were taken from *in vitro* regenerated roots growing in auxin-rich medium. On placing the explants in another auxin- rich medium may be an inhibiting factor for shoot bud differentiation while facilitating lateral root formation. This is also supported by the view that relatively high concentration of auxin suppresses the inception of shoot buds and promotes growth of lateral roots (Peterson, 1975). Cytokinins are considered to stimulate bud formation and inhibit lateral root formation. There are reports where BAP alone has found to be most efficient for shoot bud regeneration from root tissues in *Citrus mitis* (Sim *et al.,* 1989); *Citrus aurantifolia* (Bhat *et al.*, 1992); *Holostemma annulare* (Sudha *et al*., 2000); *Garnicia indica* (Deodhar *et al.*, 2000); *Blackstonia perfoliata* (Bijelovic *et al*. ,2004); *Crataeva nurvula* (Walia *et al.*,2004); *Melia azedarach* (Vila *et al.*, 2005). Contrastingly, in our study BAP when tried alone at different concentrations was found to be completely inefficient for shoot regeneration.

Wawrosch *et al.,* 1999 reported adventitious shoot regeneration from root explants of *S. chirata* where radicle explants from 3-week-old aseptically grown seedlings were used as primary explants for shoot organogenesis. They optimized a procedure of 3 weeks culturing root explants on BAP(3µM) supplemented MS medium followed by transfer to basal MS medium for another 3 weeks. However, the regeneration procedure described was not very efficient as only 1.9 shoots per explant could be achieved and the regenerated shoots were hyperhydrated. We report a significant improvement over these results as an average of 6.7 shoots per root explants could be obtained in our study which were further multiplied on MS medium supplemented with BAP, IAA and Ads and 18.0 fold multiplication was achieved. The regenerated shoots were healthy and robust without any observation of hyperhydration.

Rooting experiments were initiated after 180 days of subculturing to get maximum material for trying different hormone concentrations. Half-strength MS medium supplemented with IBA at 4.90 µM concentration proved to be most optimal for rooting. The results were similar to our previous findings on *in vitro* propagation of *S. chirata* via nodal explants. Wawrosch *et al.*(1999) had reported callus formation on exposure of shoots to media supplemented with upto 10 µM auxins for rooting. Therefore, they described a method of 2second dipping of shoots in an aqueous solution of 15 ppm NAA followed by 3-4 week cultivation period on hormone-free, half-strength MS medium for root development. In contrast, callusing did not occur in any of the treatments tried for *in vitro* rooting in our study and long, healthy root system developed on IBA augmented medium.

In vitro raised plants are heterotrophic in their mode of nutrition and cannot withstand the environmental conditions without proper hardening and acclimatization, thus, need to be hardened and acclimatized before field transplantation. Regenerated plantlets were hardened under controlled conditions of culture room on ¼ liquid MS medium and sucrose supply was decreased to 2%. After a period of one week, these plantlets were transferred to a rooting mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags in shade. Holes were made in polythene bags for gaseous

exchange. For acclimatizing the plantlets, bags were withdrawn periodically. During the process, the shoots elongated, leaves turned greener and expanded after which the polybags were completely removed. Hardened plants were subsequently transferred to soil filled in pots and over 80 % survivability was recorded.

We suggest that this ability of *S.chirata* plants of direct shoot organogenesis via root in nature, juvenile tissues of the root and specific combinations of growth regulators collectively triggered the process of direct shoot differentiation from root explants *in vitro*.

Conclusion

This study has revealed the morphogenetic potential of *in vitro* grown roots of *S.chirata* as a source tissue for micropropagation. The explants can be easily and regularly obtained from established shoot cultures and do not require disinfection treatment hence being ideal for germplasm exchange and cryopreservation. Normal root culture establishment for a number of plant species have the ability to accumulate secondary products similar to those found in mother donor plant (Tabata *et al.*, 1972; Whitten *et al.,* 1981). In *S.chirata* root being the most important part of the plant for medicinal purposes holds importance as the starting material for tissue culture studies. The present protocol will therefore serve as alternative means for *in vitro* clonal propagation of this endangered plant with immense medicinal value and further biochemical and physiological investigations.

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De novo Shoot Organogenesis from Cultured Root Explants of *Swertia chirata* Buch.-Ham.ex Wall.: An Endangered Medicinal Plant.

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Abstract: *In vitro* regeneration of plants from root cultures of *Swertia chirata* Buch.-Ham. ex Wall was obtained. Root explants from axenic shoot cultures were used for shoot induction. Optimal shoot regeneration without any callus intervention was observed on 1/2 MS (Murashige and Skoog's medium, 1962) + 4.44 µM 6, benzylaminopurine (BAP) +1.07 µM α-naphthalene acetic acid (NAA).Regenerated shoots were further multiplied on full strength MS medium supplemented with different concentrations of plant growth regulators. Maximum shoot multiplication was achieved on MS medium fortified with 4.44 µM BAP + 2.85 µM indole-3 acetic acid (IAA) + 271.45 µM adenine sulphate (Ads). Best results of rooting were observed on 1/2 strength MS medium containing 4.90 µM IBA. Plants with well-developed shoots and roots were successfully hardened with over 80% survivability.
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Keywords: *Swertia chirata*, root culture, *de novo* organogenesis, *in vitro* plant regeneration.

Abbreviations: MS: Murashige and Skoog (1962); **BAP:** 6, Benzylaminopurine; **Kn:** 6-Furfurylaminopurine; **NAA:** α-Naphthalene Acetic Acid; **IBA:** Indole-3 butyric acid; **IAA**: Indole-3 acetic acid; **GA_3**: Gibberellic acid; **Ads:** Adenine sulphate; **PGR:** Plant growth regulator

Introduction

Swertia chirata Buch.- Ham.- commonly known as chirayata is a medicinal plant of family Gentianaceae is native to temperate Himalaya (altitude above 4,000-10,000 ft.). The plant has an erect, about 2-3 ft long stem and the whole plant is bitter in taste. *S.chirata* contains a yellow bitter ophelic acid and two bitter glucosides chiratin and amarogentin. The root is said to be the most powerful part which is included in the British and American Pharmacopoeias and is used against a variety of diseases. The bitterness, antihelmintic, hypoglycemic and antipyretic properties are attributed to amarogentin ; most bitter compound isolated till date (Karan et al., 1996), swerchirin, swertiamarin and other active principles of the herb. The whole plant contains gentiamine alkaloids and the aerial part contains xanthones. Herbal medicines such as Ayush-64, Diabecon, Mensturyl syrup and Melicon ointment contain chirata extract in different amounts (Edwin et al., 1988, Valecha et al., 2000 and Mitra et al., 1996). The drug obtained from the dried plant is held in high esteem in India and is prescribed in a variety of forms and combinations in chronic fever, anemia and is used as special remedy for bronchial asthma, liver and stomach disorders, malaria and diabetes.

Several species of the genus *Swertia* are available in India and other countries and used as adulterant. However, *S.chirata* from India *and S.japonica* and *S.pseudochinenesis* from Japan are considered elite species because of their medicinal value and revenue-generating potential.

Chirayata is difficult to propagate on mass scale via seed owing to non-availability of seeds or due to harvesting of plants before seeds mature. The species is, therefore, deprived of natural regeneration. Also, the low viability and germination percentage of seeds and the necessary delicate field handling of the seedlings are some of the factors that discourage agro technology development and commercial cultivation of the species. There is an increasing pharmaceutical demand of the species both, in the indigenous and the world market but lack of large-scale commercial plantations of the herb continues. Destructive harvesting and loss of habitat has diminished the existing populations of *S.chirata*. The species is among the highly prioritized medicinal plants of India as identified by National Medicinal Plant Board, Govt. Of India and according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria, this priority plant has been designated as critically endangered .The need to develop techniques for its mass multiplication through different regeneration pathways thus, becomes imperative.

Documented literature reveals that there are limited reports on *in vitro* propagation of *Swertia chirata.* Micropropagation via field-grown nodal explants has been reported by Ahuja *et al.* (2003), Chaudhuri *et al.* (2007), Koul *et al*. (2009) and Pant *et al.,* 2010. Joshi and Dhawan (2007 a) and Balaraju *et al.* (2009) published reports on *in vitro* propagation of *S. chirata* using shoot tip explants derived from *in vitro* grown seedlings. ISSR marker analysis of genetic diversity among *S.chirata* genotypes of temperate Himalayan region of India was done by Joshi and Dhawan (2007 b). Chaudhuri *et al.* 2008 and 2009 reported direct shoot regeneration from *in vitro* leaves regeneration via immature seed cultures of *S. chirata*. Wang *et al.* (2009) described *in vitro* shoot regeneration from leaves taken from field-grown plants.

Root tissue has been proven to be highly regenerative explant and is relatively easy to maintain and manipulate *in vitro* (Vincour *et al.*, 2000; Franklin, 2004). Besides, shoots developing on root segments of the same plant have been reported to be genetically uniform and the method, an alternative way for clonal propagation. (Ostazeski and Henson, 1965; Budd, 1973; Chaturvedi *et al.*, 1981; Sharma *et al.*, 1993). The only available report on culture of root segments of *S.chirata* procured from *in vitro* raised seedlings was by Wawrosch *et al.* (1999). However, low frequency of shoot regeneration and hyperhydration of shoots hindered the production of quality shoots employing the procedure described by them. The present report communicates a reproducible protocol for regeneration of well-developed and healthy *S. chirata* plantlets via root segment culture without any intervening callus phase. The findings will be useful in conservation and mass multiplication of this endangered medicinal plant.

Material and Methods

Explant Source

In our previous study on *S.chirata* (Pant *et al.,* 2010) the shoots were induced from nodal explants. Regenerated shoots were further multiplied , subcultured and maintained on full strength MS medium supplemented with BAP(4.44 µM). Shoots having 1 or 2 nodes were rooted on half-strength MS medium supplemented with 4.90 µM indole-3 butyric acid (IBA).Ten – weeks old *in vitro* developed roots were used as an explant material for direct shoot organogenesis. For this, *in vitro* regenerated shoots via nodal explants were rooted on half-strength MS medium supplemented with 2% sucrose and IBA (4.90 µM). Aseptically removed long roots were repeatedly washed in sterile distilled water to free them from agar adhering to the surface. Subsequently they were dissected into 2-3 cm long proximal, middle and distal segments and placed horizontally on the culture medium for shoot organogenesis.

In vitro Shoot Organogenesis

Half-strength MS medium solidified with 0.7% agar (w/v) and supplemented with 2% sucrose and varying concentrations and combinations of phytohormones viz BAP(2.22 µM-22.20 µM) and NAA(1.07 µM- 5.37 µM) was tested for direct shoot regeneration from axenic root cultures. MS medium lacking plant growth regulators served as control. Data on average shoot number per explant and average shoot length (cm) was recorded after an interval of 4 weeks.

In vitro Shoot Multiplication

Once the culture conditions for optimum shoot induction from root explants were established, the proliferated shoots were subcultured onto fresh medium of same composition to establish an initial stock of shoots. These shoots were further used to assess the effect of growth regulators on further *in vitro* shoot multiplication. Full strength MS medium agarified with 0.7% agar (w/v) and fortified with 3% sucrose and varying concentrations and combinations of plant growth regulators were used for the purpose. Experiments conducted to obtain maximum shoot multiplication largely consisted of experiments pertaining to cytokinin (4.44 µM – 13.32 µM BAP) alone and in combination with auxin (1.14 µM – 2.85 µM IAA) and/or adjuvant adenine sulphate (271.45 µM). Observations pertaining to average shoot number and average shoot length were recorded after periodic intervals of 4 weeks and 8 weeks. Routine sub culturing of multiple shoots was carried out at an interval of every 3-week.

In vitro Rooting

Experiments on *in vitro* rooting of *in vitro* grown shoots were attempted with 2-3 cm long shoots produced during shoot multiplication. Shoots were cultured in each tube on MS medium containing 2% sucrose and supplemented with auxins IAA (1.14 µM - 11.40 µM), IBA (0.98 µM - 9.80 µM) and NAA (1.07 µM - 10.74 µM). Single shoots were cultured for initiation of roots and observations were recorded after 4 weeks and 8 weeks. Rooting response was recorded in terms of average number of roots produced and average root length.

Medium and Culture conditions

The pH of medium was adjusted to 5.8 before adding 0.7% agar (w/v) and sterilized by autoclaving at 15lbs (1.8 kg/cm^2) pressure at 121^{0}C for 15 minutes. Cultures were incubated at 25 ± 1^{0}C

for 16 hours in light (illuminated by 40 watt fluorescent tubes, 50µE m^{-2} s^{-1}) and for 8 hours in dark cycle irradiance by cool fluorescent tubes.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to liquid 1/4 strength MS strength medium having 2% sucrose and devoid of any plant growth regulator, for 7 days in flasks. Plantlets with well- developed roots were then removed from culture flasks and the roots gently washed under running tap water. They were then transferred to polybags containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags and maintained in high-density double check agronet open shade house. The plantlets were suitably irrigated with tap water .The polythene bags were periodically withdrawn to acclimatize the plantlets and when new leaves started emerging the polybags were completely removed. Hardened plantlets were subsequently transferred to soil in pots for further growth and development.

Statistical Analysis

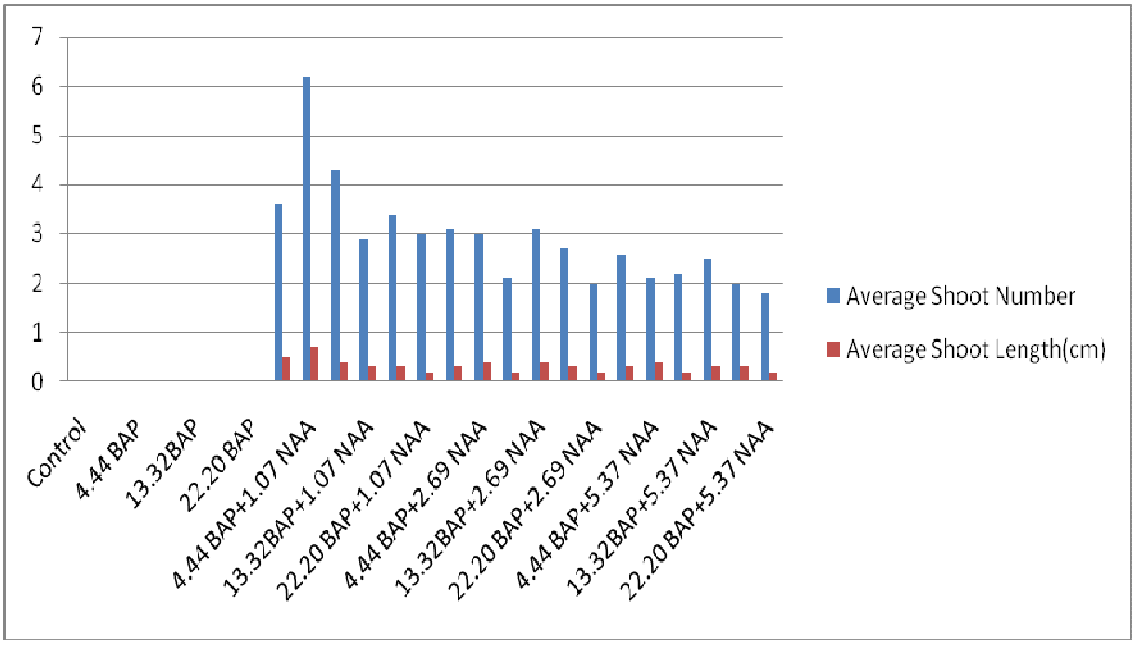
Experiments were done in triplicate and each treatment consisted of minimum ten replicates. The data was analysed using analysis of variance (ANOVA) of Completely randomized design (CRD) in GenStat Discovery Edition 13.The significance level was determined at P < = 0.05.Mean values of treatments were compared with Least Significance Difference (LSD).

Results:

***In vitro* shoot organogenesis**

De novo shoot organogenesis occurred only on proximal or middle sections of the roots. Distal regions of root explants proved to be incompetent for the purpose. Among the different treatments tried for shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) was found to be most optimal giving an average of 6.7 shoots per explant with mean shoot length of 0.7 cm without any growth of lateral roots within a period of 4 weeks [Figure 1]. MS medium without any plant growth regulator or augmented with BAP alone proved to be ineffective for shoot regeneration. On increasing the concentration of NAA to 2.69 µM and 5.37 µM , an enhanced induction of lateral roots was observed with a marked decrease in shoot bud induction from explants.(Graph 1).

Graph 1: Effect of PGR (µM) Treatment on Adventitious Shoot Regeneration from Root Explants



***In vitro* shoot multiplication**

The media combinations reported to be most optimal in our previous study on *S.chirata* (Pant *et al*., 2010) were MS medium supplemented with different concentrations of BAP, IAA and /or Ads for shoot multiplication and half-strength MS containing IBA for subsequent rooting. Similar combinations were tried in the present study and favourable results were obtained. On transferring *in vitro* grown shoots onto multiplication media, all media combinations tested were capable of inducing multiplication within 2-3 wks. Final observations in terms of mean number of shoots and shoot length were recorded after a period of 4 weeks and 8 weeks. All the treatments were significantly superior to the control except the treatments C4 and C6. In C4 (MS + 13.32 µM BAP) the average shoot number after 4 weeks and in C6 [MS+ 13.32 µM BAP+ 2.85 µM IAA] the average shoot length after 8 weeks was at par with the control. Amongst all, treatment C8 was found to be the best in all parameters. In this combination(MS + 4.44 µM BAP + 2.85 µM IAA + 271.45µM Ads) an average of 10.0 number of shoots with shoot length 2.0 cm after 4 weeks and mean shoot number 18.7 with shoot length 4.0 cm after 8 weeks was recorded [Table1] [Figure 3] .

Table 1: Effect of Plant Growth Regulators on *In vitro* Multiplication of Regenerated Microshoots

Treatment	MS medium +PGR(µM)			Average number of shoots		Average length of shoots(cm)	
	BAP	IAA	Ads	After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
C1	0.0	0.0	0.0	3.5	4	0.7	1.0
C2	4.44	0.0	0.0	4.4	7.4	1.6	2.3
C3	8.88	0.0	0.0	5.4	15	2.3	3.3
C4	13.32	0.0	0.0	5.3	9.1	1.2	1.7
C5	4.44	2.85	0.0	7.3	10.7	1.2	1.8
C6	8.88	2.85	0.0	6.4	9.8	1.1	1.6
C7	13.32	2.85	0.0	6.3	12.4	1.3	2.4
C8	**4.44**	**2.85**	**271.45**	**10.0**	**18.7**	**2.0**	**4.0**
C9	8.88	2.85	271.45	7.4	13.4	1.7	4.6
C10	13.32	2.85	271.45	8.2	11.5	1.9	2.7
Mean				6.4	11.2	1.5	2.5
LSD				1.8	2.8	0.4	1.8

After standardizing the media combination for shoot multiplication, *in vitro* multiplied shoots were subcultured on MS medium fortified with 4.44 µM BAP for maintenance of cultures.

***In vitro* rooting**

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama 1970). For the purpose, regenerated shoots were inoculated onto rooting media. It was observed that root primordia emerged from the shoot base within 10 to 14 days after shoot inoculation in all the treatments. Observations regarding mean root number and root length were recorded after 4 weeks and 8 weeks. All treatments were significantly superior to control. Optimal response was observed in the treatment R4(4.90 µM IBA) with average number of 21.0 roots per shoot after 4-weeks, 28.9 after 10-weeks and average root length 0.9 cm after 4-weeks & 1.6 cm after 10-weeks was observed in [Table 2] [Figure 4].

Table 2: Effect of Different Concentrations of IBA on Rooting of *In-vitro* Regenerated Shoots.

Treatment	1/2 strength MS medium +IBA (µM)	Average number of roots		Average length of roots(cm)	
		After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
R1	0.0	1.6	1.8	0.2	0.2
R2	0.98	6.2	12.1	0.6	1.1
R3	2.46	7.1	13.5	0.8	1.3
R4	**4.90**	**21.0**	**28.9**	**0.9**	**1.6**
R5	9.80	14.0	20.7	0.6	1.1
Mean		10.0	15.4	0.6	1.1
LSD		5.4	6.2	0.3	0.3

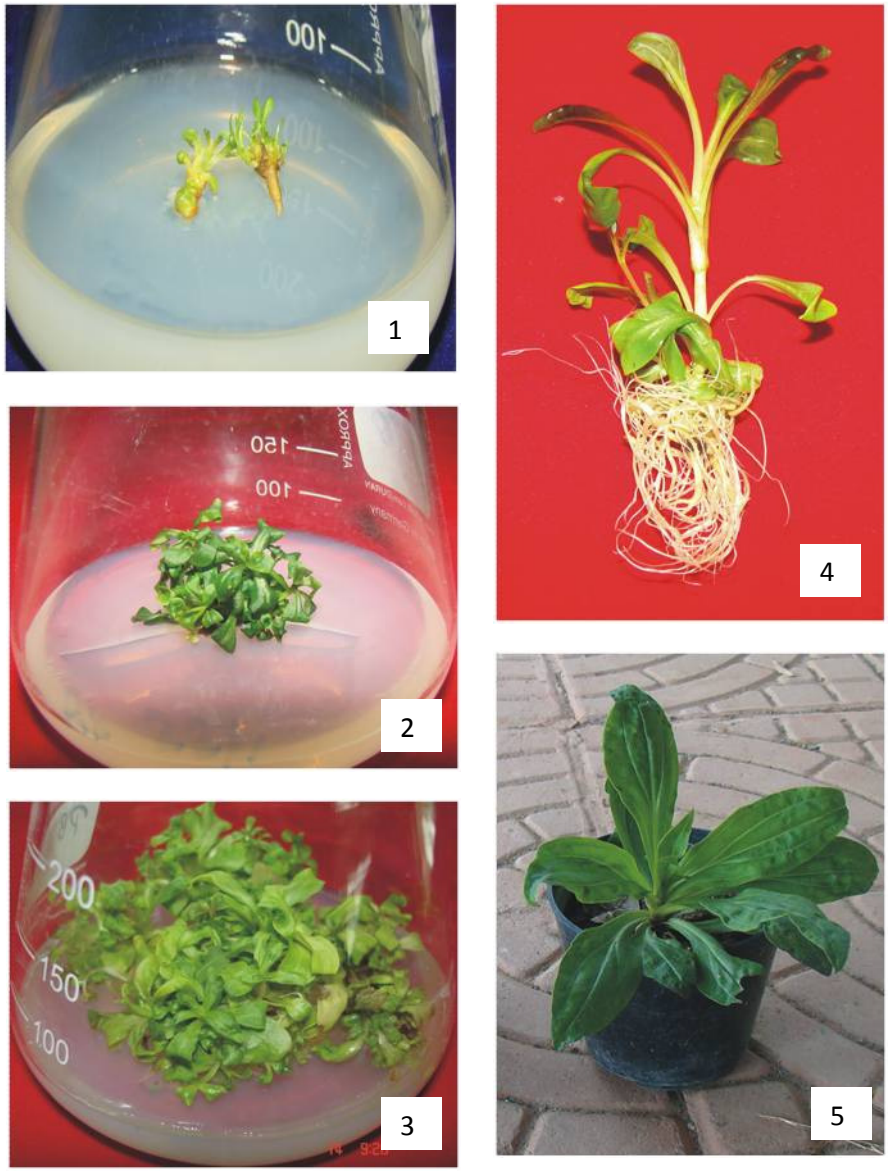


Fig. 1-5 Direct organogenesis of *Swertia chirata* via root explant:
(1) Shoot induction on root segments (2) Culture establishment
(3) Multiplication of shoots (4) *In vitro* rooted plantlet (5) Hardened plantlet.

Hardening and acclimatization

In-vitro hardening was done on liquid 1/4MS medium having 2% sucrose, for 7 days followed by acclimatization in agronet shade house in polybags containing a mixture of soil: sand :manure (1:1:1) and covered with perforated polythene bags. These polythene bags were withdrawn periodically and on emergence of new leaves, the polybags were completely removed. The plants maintained in shade house were irrigated with water. For next one month,

plants were maintained in net house. Well-acclimatized plants were shifted to pots containing soil and gave percentage survival of over 80%. [Figure 5].

Discussion

Careful observations revealed that of the different root segments tried for shoot bud initiation in our study, distal root segments remained non-responsive to shoot bud initiation while proximal and middle segments exhibited shoot regeneration and apparently no difference in the frequency and mode of shoot bud differentiation. Bud initiation and its formation uniformly from the proximal or middle portion of the root indicate that some secondary growth may also be essential for shoot initiation. The results are in corroboration with reports on other species where only the proximal and middle root sections exhibited *de novo* shoot organogenesis in *Comptonia peregrine* (Goforth and Torrey,1977), *Brassica napus* (Sharma and Thorpe, 1987), *Holostemma annulare* (Sudha *et al.*, 2000), *Populus tremula* (Vinocur *et al*.,2000), *Hypericum perforatum* (Zobayed and Saxena, 2003).

In the present study, among the different treatments tried for *in vitro* shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) gave maximum number of shoots without any growth of lateral roots within a period of 3 weeks . A synergistic effect of BAP and NAA has been highlighted in previous studies on shoot initiation from root explants of *Dalbergia sissoo* (Mukhopadhay and Mohan Ram,1981); *Citrus sinensis* (Burger and Hackett, 1986); *Citrus mitis* (Sim *et al.*, 1989); *Averrhoa carambola* (Kantharajah *et al.*,1992); *Clitoria ternatea* (Shahzad *et al.,*2007) and *Podophyllum hexandrum* (Chakraborty *et al*. ,2010) .

However, in our study, on increasing the concentration of NAA to 2.69 µM and 5.37 µM, significant reduction in shoot bud initiation was observed with an enhanced induction of lateral roots from cut end of roots. Root explants in our study were taken from *in vitro* regenerated roots growing in auxin-rich medium. On placing the explants in another auxin- rich medium may be an inhibiting factor for shoot bud differentiation while facilitating lateral root formation. This is also supported by the view that relatively high concentration of auxin suppresses the inception of shoot buds and promotes growth of lateral roots (Peterson, 1975). Cytokinins are considered to stimulate bud formation and inhibit lateral root formation. There are reports where BAP alone has found to be most efficient for shoot bud regeneration from root tissues in *Citrus mitis* (Sim *et al.,* 1989); *Citrus aurantifolia* (Bhat *et al.*, 1992); *Holostemma annulare* (Sudha *et al*., 2000); *Garnicia indica* (Deodhar *et al.*, 2000); *Blackstonia perfoliata* (Bijelovic *et al*. ,2004); *Crataeva nurvula* (Walia *et al.*,2004); *Melia azedarach* (Vila *et al.*, 2005). Contrastingly, in our study BAP when tried alone at different concentrations was found to be completely inefficient for shoot regeneration.

Wawrosch *et al.,* 1999 reported adventitious shoot regeneration from root explants of *S. chirata* where radicle explants from 3-week-old aseptically grown seedlings were used as primary explants for shoot organogenesis. They optimized a procedure of 3 weeks culturing root explants on BAP(3µM) supplemented MS medium followed by transfer to basal MS medium for another 3 weeks. However, the regeneration procedure described was not very efficient as only 1.9 shoots per explant could be achieved and the regenerated shoots were hyperhydrated. We report a significant improvement over these results as an average of 6.7 shoots per root explants could be obtained in our study which were further multiplied on MS medium supplemented with BAP, IAA and Ads and 18.0 fold multiplication was achieved. The regenerated shoots were healthy and robust without any observation of hyperhydration.

Rooting experiments were initiated after 180 days of subculturing to get maximum material for trying different hormone concentrations. Half-strength MS medium supplemented with IBA at 4.90 µM concentration proved to be most optimal for rooting. The results were similar to our previous findings on *in vitro* propagation of *S. chirata* via nodal explants. Wawrosch *et al.*(1999) had reported callus formation on exposure of shoots to media supplemented with upto 10 µM auxins for rooting. Therefore, they described a method of 2second dipping of shoots in an aqueous solution of 15 ppm NAA followed by 3-4 week cultivation period on hormone-free, half-strength MS medium for root development. In contrast, callusing did not occur in any of the treatments tried for *in vitro* rooting in our study and long, healthy root system developed on IBA augmented medium.

In vitro raised plants are heterotrophic in their mode of nutrition and cannot withstand the environmental conditions without proper hardening and acclimatization, thus, need to be hardened and acclimatized before field transplantation. Regenerated plantlets were hardened under controlled conditions of culture room on ¼ liquid MS medium and sucrose supply was decreased to 2%. After a period of one week, these plantlets were transferred to a rooting mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags in shade. Holes were made in polythene bags for gaseous

exchange. For acclimatizing the plantlets, bags were withdrawn periodically. During the process, the shoots elongated, leaves turned greener and expanded after which the polybags were completely removed. Hardened plants were subsequently transferred to soil filled in pots and over 80 % survivability was recorded.

We suggest that this ability of *S.chirata* plants of direct shoot organogenesis via root in nature, juvenile tissues of the root and specific combinations of growth regulators collectively triggered the process of direct shoot differentiation from root explants *in vitro*.

Conclusion

This study has revealed the morphogenetic potential of *in vitro* grown roots of *S.chirata* as a source tissue for micropropagation. The explants can be easily and regularly obtained from established shoot cultures and do not require disinfection treatment hence being ideal for germplasm exchange and cryopreservation. Normal root culture establishment for a number of plant species have the ability to accumulate secondary products similar to those found in mother donor plant (Tabata *et al.*, 1972; Whitten *et al.,* 1981). In *S.chirata* root being the most important part of the plant for medicinal purposes holds importance as the starting material for tissue culture studies. The present protocol will therefore serve as alternative means for *in vitro* clonal propagation of this endangered plant with immense medicinal value and further biochemical and physiological investigations.

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De novo Shoot Organogenesis from Cultured Root Explants of *Swertia chirata* Buch.-Ham.ex Wall.: An Endangered Medicinal Plant.

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Abstract: *In vitro* regeneration of plants from root cultures of *Swertia chirata* Buch.-Ham. ex Wall was obtained. Root explants from axenic shoot cultures were used for shoot induction. Optimal shoot regeneration without any callus intervention was observed on 1/2 MS (Murashige and Skoog's medium, 1962) + 4.44 µM 6, benzylaminopurine (BAP) +1.07 µM α-naphthalene acetic acid (NAA).Regenerated shoots were further multiplied on full strength MS medium supplemented with different concentrations of plant growth regulators. Maximum shoot multiplication was achieved on MS medium fortified with 4.44 µM BAP + 2.85 µM indole-3 acetic acid (IAA) + 271.45 µM adenine sulphate (Ads). Best results of rooting were observed on 1/2 strength MS medium containing 4.90 µM IBA. Plants with well-developed shoots and roots were successfully hardened with over 80% survivability.
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Keywords: *Swertia chirata*, root culture, *de novo* organogenesis, *in vitro* plant regeneration.

Abbreviations: MS: Murashige and Skoog (1962); **BAP:** 6, Benzylaminopurine; **Kn:** 6-Furfurylaminopurine; **NAA:** α-Naphthalene Acetic Acid; **IBA:** Indole-3 butyric acid; **IAA**: Indole-3 acetic acid; **GA_3**: Gibberellic acid; **Ads:** Adenine sulphate; **PGR:** Plant growth regulator

Introduction

Swertia chirata Buch.- Ham.- commonly known as chirayata is a medicinal plant of family Gentianaceae is native to temperate Himalaya (altitude above 4,000-10,000 ft.). The plant has an erect, about 2-3 ft long stem and the whole plant is bitter in taste. *S.chirata* contains a yellow bitter ophelic acid and two bitter glucosides chiratin and amarogentin. The root is said to be the most powerful part which is included in the British and American Pharmacopoeias and is used against a variety of diseases. The bitterness, antihelmintic, hypoglycemic and antipyretic properties are attributed to amarogentin ; most bitter compound isolated till date (Karan et al., 1996), swerchirin, swertiamarin and other active principles of the herb. The whole plant contains gentiamine alkaloids and the aerial part contains xanthones. Herbal medicines such as Ayush-64, Diabecon, Mensturyl syrup and Melicon ointment contain chirata extract in different amounts (Edwin et al., 1988, Valecha et al., 2000 and Mitra et al., 1996). The drug obtained from the dried plant is held in high esteem in India and is prescribed in a variety of forms and combinations in chronic fever, anemia and is used as special remedy for bronchial asthma, liver and stomach disorders, malaria and diabetes.

Several species of the genus *Swertia* are available in India and other countries and used as adulterant. However, *S.chirata* from India *and S.japonica* and *S.pseudochinenesis* from Japan are considered elite species because of their medicinal value and revenue-generating potential.

Chirayata is difficult to propagate on mass scale via seed owing to non-availability of seeds or due to harvesting of plants before seeds mature. The species is, therefore, deprived of natural regeneration. Also, the low viability and germination percentage of seeds and the necessary delicate field handling of the seedlings are some of the factors that discourage agro technology development and commercial cultivation of the species. There is an increasing pharmaceutical demand of the species both, in the indigenous and the world market but lack of large-scale commercial plantations of the herb continues. Destructive harvesting and loss of habitat has diminished the existing populations of *S.chirata*. The species is among the highly prioritized medicinal plants of India as identified by National Medicinal Plant Board, Govt. Of India and according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria, this priority plant has been designated as critically endangered .The need to develop techniques for its mass multiplication through different regeneration pathways thus, becomes imperative.

Documented literature reveals that there are limited reports on *in vitro* propagation of *Swertia chirata.* Micropropagation via field-grown nodal explants has been reported by Ahuja *et al.* (2003), Chaudhuri *et al.* (2007), Koul *et al*. (2009) and Pant *et al.,* 2010. Joshi and Dhawan (2007 a) and Balaraju *et al.* (2009) published reports on *in vitro* propagation of *S. chirata* using shoot tip explants derived from *in vitro* grown seedlings. ISSR marker analysis of genetic diversity among *S.chirata* genotypes of temperate Himalayan region of India was done by Joshi and Dhawan (2007 b). Chaudhuri *et al.* 2008 and 2009 reported direct shoot regeneration from *in vitro* leaves regeneration via immature seed cultures of *S. chirata*. Wang *et al.* (2009) described *in vitro* shoot regeneration from leaves taken from field-grown plants.

Root tissue has been proven to be highly regenerative explant and is relatively easy to maintain and manipulate *in vitro* (Vincour *et al.*, 2000; Franklin, 2004). Besides, shoots developing on root segments of the same plant have been reported to be genetically uniform and the method, an alternative way for clonal propagation. (Ostazeski and Henson, 1965; Budd, 1973; Chaturvedi *et al.*, 1981; Sharma *et al.*, 1993). The only available report on culture of root segments of *S.chirata* procured from *in vitro* raised seedlings was by Wawrosch *et al.* (1999). However, low frequency of shoot regeneration and hyperhydration of shoots hindered the production of quality shoots employing the procedure described by them. The present report communicates a reproducible protocol for regeneration of well-developed and healthy *S. chirata* plantlets via root segment culture without any intervening callus phase. The findings will be useful in conservation and mass multiplication of this endangered medicinal plant.

Material and Methods

Explant Source

In our previous study on *S.chirata* (Pant *et al.,* 2010) the shoots were induced from nodal explants. Regenerated shoots were further multiplied , subcultured and maintained on full strength MS medium supplemented with BAP(4.44 µM). Shoots having 1 or 2 nodes were rooted on half-strength MS medium supplemented with 4.90 µM indole-3 butyric acid (IBA).Ten – weeks old *in vitro* developed roots were used as an explant material for direct shoot organogenesis. For this, *in vitro* regenerated shoots via nodal explants were rooted on half-strength MS medium supplemented with 2% sucrose and IBA (4.90 µM). Aseptically removed long roots were repeatedly washed in sterile distilled water to free them from agar adhering to the surface. Subsequently they were dissected into 2-3 cm long proximal, middle and distal segments and placed horizontally on the culture medium for shoot organogenesis.

In vitro Shoot Organogenesis

Half-strength MS medium solidified with 0.7% agar (w/v) and supplemented with 2% sucrose and varying concentrations and combinations of phytohormones viz BAP(2.22 µM-22.20 µM) and NAA(1.07 µM- 5.37 µM) was tested for direct shoot regeneration from axenic root cultures. MS medium lacking plant growth regulators served as control. Data on average shoot number per explant and average shoot length (cm) was recorded after an interval of 4 weeks.

In vitro Shoot Multiplication

Once the culture conditions for optimum shoot induction from root explants were established, the proliferated shoots were subcultured onto fresh medium of same composition to establish an initial stock of shoots. These shoots were further used to assess the effect of growth regulators on further *in vitro* shoot multiplication. Full strength MS medium agarified with 0.7% agar (w/v) and fortified with 3% sucrose and varying concentrations and combinations of plant growth regulators were used for the purpose. Experiments conducted to obtain maximum shoot multiplication largely consisted of experiments pertaining to cytokinin (4.44 µM – 13.32 µM BAP) alone and in combination with auxin (1.14 µM – 2.85 µM IAA) and/or adjuvant adenine sulphate (271.45 µM). Observations pertaining to average shoot number and average shoot length were recorded after periodic intervals of 4 weeks and 8 weeks. Routine sub culturing of multiple shoots was carried out at an interval of every 3-week.

In vitro Rooting

Experiments on *in vitro* rooting of *in vitro* grown shoots were attempted with 2-3 cm long shoots produced during shoot multiplication. Shoots were cultured in each tube on MS medium containing 2% sucrose and supplemented with auxins IAA (1.14 µM - 11.40 µM), IBA (0.98 µM - 9.80 µM) and NAA (1.07 µM - 10.74 µM). Single shoots were cultured for initiation of roots and observations were recorded after 4 weeks and 8 weeks. Rooting response was recorded in terms of average number of roots produced and average root length.

Medium and Culture conditions

The pH of medium was adjusted to 5.8 before adding 0.7% agar (w/v) and sterilized by autoclaving at 15lbs (1.8 kg/cm^2) pressure at 121^{0}C for 15 minutes. Cultures were incubated at 25 ± 1^{0}C

for 16 hours in light (illuminated by 40 watt fluorescent tubes, 50µE m^{-2} s^{-1}) and for 8 hours in dark cycle irradiance by cool fluorescent tubes.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to liquid 1/4 strength MS strength medium having 2% sucrose and devoid of any plant growth regulator, for 7 days in flasks. Plantlets with well- developed roots were then removed from culture flasks and the roots gently washed under running tap water. They were then transferred to polybags containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags and maintained in high-density double check agronet open shade house. The plantlets were suitably irrigated with tap water .The polythene bags were periodically withdrawn to acclimatize the plantlets and when new leaves started emerging the polybags were completely removed. Hardened plantlets were subsequently transferred to soil in pots for further growth and development.

Statistical Analysis

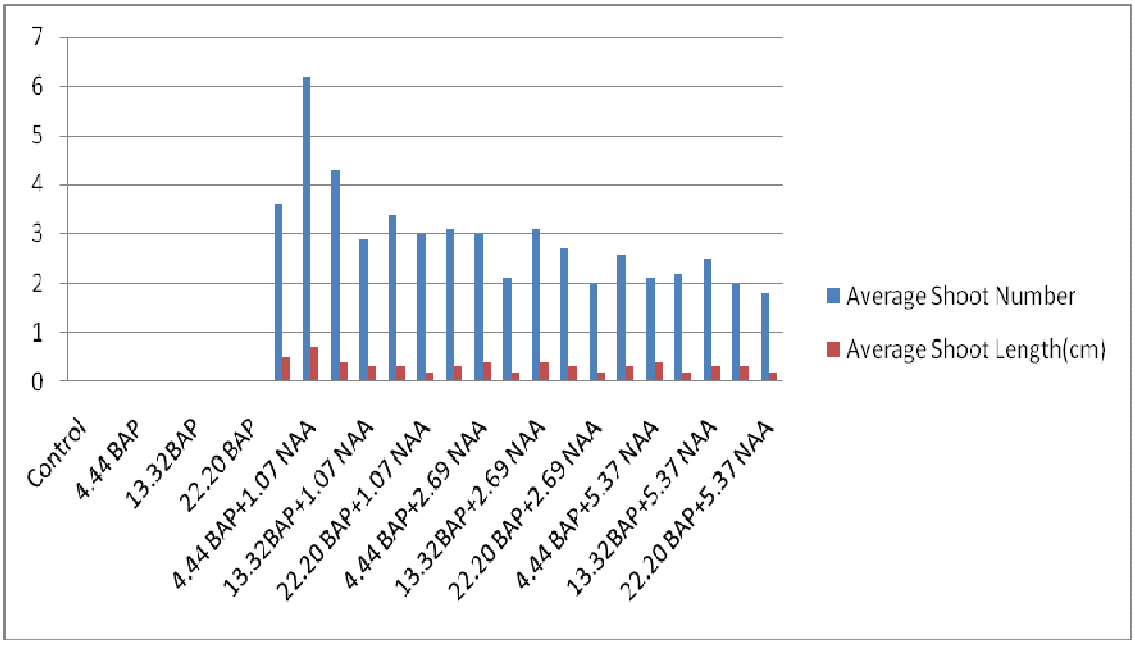
Experiments were done in triplicate and each treatment consisted of minimum ten replicates. The data was analysed using analysis of variance (ANOVA) of Completely randomized design (CRD) in GenStat Discovery Edition 13.The significance level was determined at P < = 0.05.Mean values of treatments were compared with Least Significance Difference (LSD).

Results:

***In vitro* shoot organogenesis**

De novo shoot organogenesis occurred only on proximal or middle sections of the roots. Distal regions of root explants proved to be incompetent for the purpose. Among the different treatments tried for shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) was found to be most optimal giving an average of 6.7 shoots per explant with mean shoot length of 0.7 cm without any growth of lateral roots within a period of 4 weeks [Figure 1]. MS medium without any plant growth regulator or augmented with BAP alone proved to be ineffective for shoot regeneration. On increasing the concentration of NAA to 2.69 µM and 5.37 µM , an enhanced induction of lateral roots was observed with a marked decrease in shoot bud induction from explants.(Graph 1).

Graph 1: Effect of PGR (µM) Treatment on Adventitious Shoot Regeneration from Root Explants



***In vitro* shoot multiplication**

The media combinations reported to be most optimal in our previous study on *S.chirata* (Pant *et al*., 2010) were MS medium supplemented with different concentrations of BAP, IAA and /or Ads for shoot multiplication and half-strength MS containing IBA for subsequent rooting. Similar combinations were tried in the present study and favourable results were obtained. On transferring *in vitro* grown shoots onto multiplication media, all media combinations tested were capable of inducing multiplication within 2-3 wks. Final observations in terms of mean number of shoots and shoot length were recorded after a period of 4 weeks and 8 weeks. All the treatments were significantly superior to the control except the treatments C4 and C6. In C4 (MS + 13.32 µM BAP) the average shoot number after 4 weeks and in C6 [MS+ 13.32 µM BAP+ 2.85 µM IAA] the average shoot length after 8 weeks was at par with the control. Amongst all, treatment C8 was found to be the best in all parameters. In this combination(MS + 4.44 µM BAP + 2.85 µM IAA + 271.45µM Ads) an average of 10.0 number of shoots with shoot length 2.0 cm after 4 weeks and mean shoot number 18.7 with shoot length 4.0 cm after 8 weeks was recorded [Table1] [Figure 3] .

Table 1: Effect of Plant Growth Regulators on *In vitro* Multiplication of Regenerated Microshoots

Treatment	MS medium +PGR(µM)			Average number of shoots		Average length of shoots(cm)	
	BAP	IAA	Ads	After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
C1	0.0	0.0	0.0	3.5	4	0.7	1.0
C2	4.44	0.0	0.0	4.4	7.4	1.6	2.3
C3	8.88	0.0	0.0	5.4	15	2.3	3.3
C4	13.32	0.0	0.0	5.3	9.1	1.2	1.7
C5	4.44	2.85	0.0	7.3	10.7	1.2	1.8
C6	8.88	2.85	0.0	6.4	9.8	1.1	1.6
C7	13.32	2.85	0.0	6.3	12.4	1.3	2.4
C8	**4.44**	**2.85**	**271.45**	**10.0**	**18.7**	**2.0**	**4.0**
C9	8.88	2.85	271.45	7.4	13.4	1.7	4.6
C10	13.32	2.85	271.45	8.2	11.5	1.9	2.7
Mean				6.4	11.2	1.5	2.5
LSD				1.8	2.8	0.4	1.8

After standardizing the media combination for shoot multiplication, *in vitro* multiplied shoots were subcultured on MS medium fortified with 4.44 µM BAP for maintenance of cultures.

***In vitro* rooting**

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama 1970). For the purpose, regenerated shoots were inoculated onto rooting media. It was observed that root primordia emerged from the shoot base within 10 to 14 days after shoot inoculation in all the treatments. Observations regarding mean root number and root length were recorded after 4 weeks and 8 weeks. All treatments were significantly superior to control. Optimal response was observed in the treatment R4(4.90 µM IBA) with average number of 21.0 roots per shoot after 4-weeks, 28.9 after 10-weeks and average root length 0.9 cm after 4-weeks & 1.6 cm after 10-weeks was observed in [Table 2] [Figure 4].

Table 2: Effect of Different Concentrations of IBA on Rooting of *In-vitro* Regenerated Shoots.

Treatment	1/2 strength MS medium +IBA (µM)	Average number of roots		Average length of roots(cm)	
		After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
R1	0.0	1.6	1.8	0.2	0.2
R2	0.98	6.2	12.1	0.6	1.1
R3	2.46	7.1	13.5	0.8	1.3
R4	**4.90**	**21.0**	**28.9**	**0.9**	**1.6**
R5	9.80	14.0	20.7	0.6	1.1
Mean		10.0	15.4	0.6	1.1
LSD		5.4	6.2	0.3	0.3

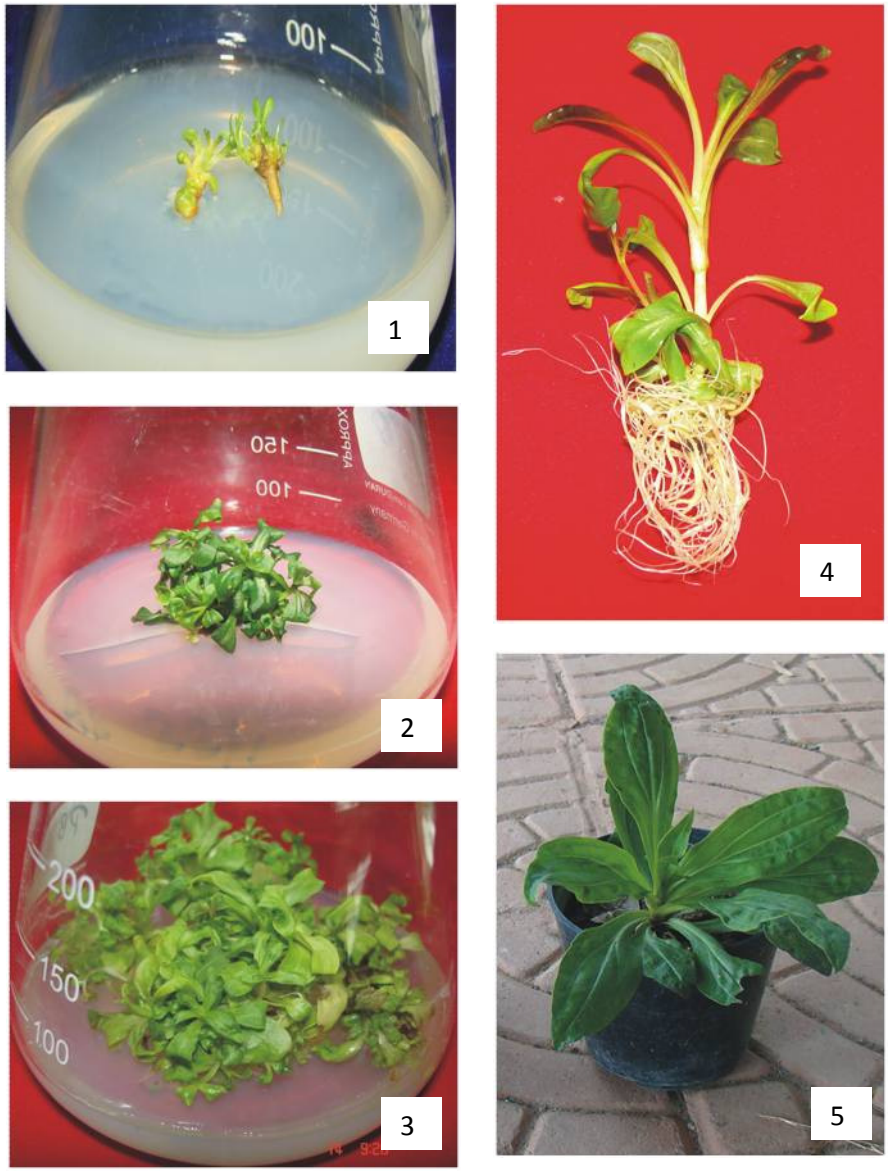


Fig. 1-5 Direct organogenesis of *Swertia chirata* via root explant:
(1) Shoot induction on root segments (2) Culture establishment
(3) Multiplication of shoots (4) *In vitro* rooted plantlet (5) Hardened plantlet.

Hardening and acclimatization

In-vitro hardening was done on liquid 1/4MS medium having 2% sucrose, for 7 days followed by acclimatization in agronet shade house in polybags containing a mixture of soil: sand :manure (1:1:1) and covered with perforated polythene bags. These polythene bags were withdrawn periodically and on emergence of new leaves, the polybags were completely removed. The plants maintained in shade house were irrigated with water. For next one month,

plants were maintained in net house. Well-acclimatized plants were shifted to pots containing soil and gave percentage survival of over 80%. [Figure 5].

Discussion

Careful observations revealed that of the different root segments tried for shoot bud initiation in our study, distal root segments remained non-responsive to shoot bud initiation while proximal and middle segments exhibited shoot regeneration and apparently no difference in the frequency and mode of shoot bud differentiation. Bud initiation and its formation uniformly from the proximal or middle portion of the root indicate that some secondary growth may also be essential for shoot initiation. The results are in corroboration with reports on other species where only the proximal and middle root sections exhibited *de novo* shoot organogenesis in *Comptonia peregrine* (Goforth and Torrey,1977), *Brassica napus* (Sharma and Thorpe, 1987), *Holostemma annulare* (Sudha *et al.*, 2000), *Populus tremula* (Vinocur *et al*.,2000), *Hypericum perforatum* (Zobayed and Saxena, 2003).

In the present study, among the different treatments tried for *in vitro* shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) gave maximum number of shoots without any growth of lateral roots within a period of 3 weeks . A synergistic effect of BAP and NAA has been highlighted in previous studies on shoot initiation from root explants of *Dalbergia sissoo* (Mukhopadhay and Mohan Ram,1981); *Citrus sinensis* (Burger and Hackett, 1986); *Citrus mitis* (Sim *et al.*, 1989); *Averrhoa carambola* (Kantharajah *et al.*,1992); *Clitoria ternatea* (Shahzad *et al.,*2007) and *Podophyllum hexandrum* (Chakraborty *et al*. ,2010) .

However, in our study, on increasing the concentration of NAA to 2.69 µM and 5.37 µM, significant reduction in shoot bud initiation was observed with an enhanced induction of lateral roots from cut end of roots. Root explants in our study were taken from *in vitro* regenerated roots growing in auxin-rich medium. On placing the explants in another auxin- rich medium may be an inhibiting factor for shoot bud differentiation while facilitating lateral root formation. This is also supported by the view that relatively high concentration of auxin suppresses the inception of shoot buds and promotes growth of lateral roots (Peterson, 1975). Cytokinins are considered to stimulate bud formation and inhibit lateral root formation. There are reports where BAP alone has found to be most efficient for shoot bud regeneration from root tissues in *Citrus mitis* (Sim *et al.,* 1989); *Citrus aurantifolia* (Bhat *et al.*, 1992); *Holostemma annulare* (Sudha *et al*., 2000); *Garnicia indica* (Deodhar *et al.*, 2000); *Blackstonia perfoliata* (Bijelovic *et al*. ,2004); *Crataeva nurvula* (Walia *et al.*,2004); *Melia azedarach* (Vila *et al.*, 2005). Contrastingly, in our study BAP when tried alone at different concentrations was found to be completely inefficient for shoot regeneration.

Wawrosch *et al.,* 1999 reported adventitious shoot regeneration from root explants of *S. chirata* where radicle explants from 3-week-old aseptically grown seedlings were used as primary explants for shoot organogenesis. They optimized a procedure of 3 weeks culturing root explants on BAP(3µM) supplemented MS medium followed by transfer to basal MS medium for another 3 weeks. However, the regeneration procedure described was not very efficient as only 1.9 shoots per explant could be achieved and the regenerated shoots were hyperhydrated. We report a significant improvement over these results as an average of 6.7 shoots per root explants could be obtained in our study which were further multiplied on MS medium supplemented with BAP, IAA and Ads and 18.0 fold multiplication was achieved. The regenerated shoots were healthy and robust without any observation of hyperhydration.

Rooting experiments were initiated after 180 days of subculturing to get maximum material for trying different hormone concentrations. Half-strength MS medium supplemented with IBA at 4.90 µM concentration proved to be most optimal for rooting. The results were similar to our previous findings on *in vitro* propagation of *S. chirata* via nodal explants. Wawrosch *et al.*(1999) had reported callus formation on exposure of shoots to media supplemented with upto 10 µM auxins for rooting. Therefore, they described a method of 2second dipping of shoots in an aqueous solution of 15 ppm NAA followed by 3-4 week cultivation period on hormone-free, half-strength MS medium for root development. In contrast, callusing did not occur in any of the treatments tried for *in vitro* rooting in our study and long, healthy root system developed on IBA augmented medium.

In vitro raised plants are heterotrophic in their mode of nutrition and cannot withstand the environmental conditions without proper hardening and acclimatization, thus, need to be hardened and acclimatized before field transplantation. Regenerated plantlets were hardened under controlled conditions of culture room on ¼ liquid MS medium and sucrose supply was decreased to 2%. After a period of one week, these plantlets were transferred to a rooting mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags in shade. Holes were made in polythene bags for gaseous

exchange. For acclimatizing the plantlets, bags were withdrawn periodically. During the process, the shoots elongated, leaves turned greener and expanded after which the polybags were completely removed. Hardened plants were subsequently transferred to soil filled in pots and over 80 % survivability was recorded.

We suggest that this ability of *S.chirata* plants of direct shoot organogenesis via root in nature, juvenile tissues of the root and specific combinations of growth regulators collectively triggered the process of direct shoot differentiation from root explants *in vitro*.

Conclusion

This study has revealed the morphogenetic potential of *in vitro* grown roots of *S.chirata* as a source tissue for micropropagation. The explants can be easily and regularly obtained from established shoot cultures and do not require disinfection treatment hence being ideal for germplasm exchange and cryopreservation. Normal root culture establishment for a number of plant species have the ability to accumulate secondary products similar to those found in mother donor plant (Tabata *et al.*, 1972; Whitten *et al.,* 1981). In *S.chirata* root being the most important part of the plant for medicinal purposes holds importance as the starting material for tissue culture studies. The present protocol will therefore serve as alternative means for *in vitro* clonal propagation of this endangered plant with immense medicinal value and further biochemical and physiological investigations.

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De novo Shoot Organogenesis from Cultured Root Explants of *Swertia chirata* Buch.-Ham.ex Wall.: An Endangered Medicinal Plant.

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Abstract: *In vitro* regeneration of plants from root cultures of *Swertia chirata* Buch.-Ham. ex Wall was obtained. Root explants from axenic shoot cultures were used for shoot induction. Optimal shoot regeneration without any callus intervention was observed on 1/2 MS (Murashige and Skoog's medium, 1962) + 4.44 µM 6, benzylaminopurine (BAP) +1.07 µM α-naphthalene acetic acid (NAA).Regenerated shoots were further multiplied on full strength MS medium supplemented with different concentrations of plant growth regulators. Maximum shoot multiplication was achieved on MS medium fortified with 4.44 µM BAP + 2.85 µM indole-3 acetic acid (IAA) + 271.45 µM adenine sulphate (Ads). Best results of rooting were observed on 1/2 strength MS medium containing 4.90 µM IBA. Plants with well-developed shoots and roots were successfully hardened with over 80% survivability.
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Keywords: *Swertia chirata*, root culture, *de novo* organogenesis, *in vitro* plant regeneration.

Abbreviations: MS: Murashige and Skoog (1962); **BAP:** 6, Benzylaminopurine; **Kn:** 6-Furfurylaminopurine; **NAA:** α-Naphthalene Acetic Acid; **IBA:** Indole-3 butyric acid; **IAA**: Indole-3 acetic acid; **GA_3**: Gibberellic acid; **Ads:** Adenine sulphate; **PGR:** Plant growth regulator

Introduction

Swertia chirata Buch.- Ham.- commonly known as chirayata is a medicinal plant of family Gentianaceae is native to temperate Himalaya (altitude above 4,000-10,000 ft.). The plant has an erect, about 2-3 ft long stem and the whole plant is bitter in taste. *S.chirata* contains a yellow bitter ophelic acid and two bitter glucosides chiratin and amarogentin. The root is said to be the most powerful part which is included in the British and American Pharmacopoeias and is used against a variety of diseases. The bitterness, antihelmintic, hypoglycemic and antipyretic properties are attributed to amarogentin ; most bitter compound isolated till date (Karan et al., 1996), swerchirin, swertiamarin and other active principles of the herb. The whole plant contains gentiamine alkaloids and the aerial part contains xanthones. Herbal medicines such as Ayush-64, Diabecon, Mensturyl syrup and Melicon ointment contain chirata extract in different amounts (Edwin et al., 1988, Valecha et al., 2000 and Mitra et al., 1996). The drug obtained from the dried plant is held in high esteem in India and is prescribed in a variety of forms and combinations in chronic fever, anemia and is used as special remedy for bronchial asthma, liver and stomach disorders, malaria and diabetes.

Several species of the genus *Swertia* are available in India and other countries and used as adulterant. However, *S.chirata* from India *and S.japonica* and *S.pseudochinenesis* from Japan are considered elite species because of their medicinal value and revenue-generating potential.

Chirayata is difficult to propagate on mass scale via seed owing to non-availability of seeds or due to harvesting of plants before seeds mature. The species is, therefore, deprived of natural regeneration. Also, the low viability and germination percentage of seeds and the necessary delicate field handling of the seedlings are some of the factors that discourage agro technology development and commercial cultivation of the species. There is an increasing pharmaceutical demand of the species both, in the indigenous and the world market but lack of large-scale commercial plantations of the herb continues. Destructive harvesting and loss of habitat has diminished the existing populations of *S.chirata*. The species is among the highly prioritized medicinal plants of India as identified by National Medicinal Plant Board, Govt. Of India and according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria, this priority plant has been designated as critically endangered .The need to develop techniques for its mass multiplication through different regeneration pathways thus, becomes imperative.

Documented literature reveals that there are limited reports on *in vitro* propagation of *Swertia chirata.* Micropropagation via field-grown nodal explants has been reported by Ahuja *et al.* (2003), Chaudhuri *et al.* (2007), Koul *et al*. (2009) and Pant *et al.,* 2010. Joshi and Dhawan (2007 a) and Balaraju *et al.* (2009) published reports on *in vitro* propagation of *S. chirata* using shoot tip explants derived from *in vitro* grown seedlings. ISSR marker analysis of genetic diversity among *S.chirata* genotypes of temperate Himalayan region of India was done by Joshi and Dhawan (2007 b). Chaudhuri *et al.* 2008 and 2009 reported direct shoot regeneration from *in vitro* leaves regeneration via immature seed cultures of *S. chirata*. Wang *et al.* (2009) described *in vitro* shoot regeneration from leaves taken from field-grown plants.

Root tissue has been proven to be highly regenerative explant and is relatively easy to maintain and manipulate *in vitro* (Vincour *et al.*, 2000; Franklin, 2004). Besides, shoots developing on root segments of the same plant have been reported to be genetically uniform and the method, an alternative way for clonal propagation. (Ostazeski and Henson, 1965; Budd, 1973; Chaturvedi *et al.*, 1981; Sharma *et al.*, 1993). The only available report on culture of root segments of *S.chirata* procured from *in vitro* raised seedlings was by Wawrosch *et al.* (1999). However, low frequency of shoot regeneration and hyperhydration of shoots hindered the production of quality shoots employing the procedure described by them. The present report communicates a reproducible protocol for regeneration of well-developed and healthy *S. chirata* plantlets via root segment culture without any intervening callus phase. The findings will be useful in conservation and mass multiplication of this endangered medicinal plant.

Material and Methods

Explant Source

In our previous study on *S.chirata* (Pant *et al.,* 2010) the shoots were induced from nodal explants. Regenerated shoots were further multiplied , subcultured and maintained on full strength MS medium supplemented with BAP(4.44 µM). Shoots having 1 or 2 nodes were rooted on half-strength MS medium supplemented with 4.90 µM indole-3 butyric acid (IBA).Ten – weeks old *in vitro* developed roots were used as an explant material for direct shoot organogenesis. For this, *in vitro* regenerated shoots via nodal explants were rooted on half-strength MS medium supplemented with 2% sucrose and IBA (4.90 µM). Aseptically removed long roots were repeatedly washed in sterile distilled water to free them from agar adhering to the surface. Subsequently they were dissected into 2-3 cm long proximal, middle and distal segments and placed horizontally on the culture medium for shoot organogenesis.

In vitro Shoot Organogenesis

Half-strength MS medium solidified with 0.7% agar (w/v) and supplemented with 2% sucrose and varying concentrations and combinations of phytohormones viz BAP(2.22 µM-22.20 µM) and NAA(1.07 µM- 5.37 µM) was tested for direct shoot regeneration from axenic root cultures. MS medium lacking plant growth regulators served as control. Data on average shoot number per explant and average shoot length (cm) was recorded after an interval of 4 weeks.

In vitro Shoot Multiplication

Once the culture conditions for optimum shoot induction from root explants were established, the proliferated shoots were subcultured onto fresh medium of same composition to establish an initial stock of shoots. These shoots were further used to assess the effect of growth regulators on further *in vitro* shoot multiplication. Full strength MS medium agarified with 0.7% agar (w/v) and fortified with 3% sucrose and varying concentrations and combinations of plant growth regulators were used for the purpose. Experiments conducted to obtain maximum shoot multiplication largely consisted of experiments pertaining to cytokinin (4.44 µM – 13.32 µM BAP) alone and in combination with auxin (1.14 µM – 2.85 µM IAA) and/or adjuvant adenine sulphate (271.45 µM). Observations pertaining to average shoot number and average shoot length were recorded after periodic intervals of 4 weeks and 8 weeks. Routine sub culturing of multiple shoots was carried out at an interval of every 3-week.

In vitro Rooting

Experiments on *in vitro* rooting of *in vitro* grown shoots were attempted with 2-3 cm long shoots produced during shoot multiplication. Shoots were cultured in each tube on MS medium containing 2% sucrose and supplemented with auxins IAA (1.14 µM - 11.40 µM), IBA (0.98 µM - 9.80 µM) and NAA (1.07 µM - 10.74 µM). Single shoots were cultured for initiation of roots and observations were recorded after 4 weeks and 8 weeks. Rooting response was recorded in terms of average number of roots produced and average root length.

Medium and Culture conditions

The pH of medium was adjusted to 5.8 before adding 0.7% agar (w/v) and sterilized by autoclaving at 15lbs (1.8 kg/cm^2) pressure at 121^{0}C for 15 minutes. Cultures were incubated at 25 ± 1^{0}C

for 16 hours in light (illuminated by 40 watt fluorescent tubes, 50µE m^{-2} s^{-1}) and for 8 hours in dark cycle irradiance by cool fluorescent tubes.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to liquid 1/4 strength MS strength medium having 2% sucrose and devoid of any plant growth regulator, for 7 days in flasks. Plantlets with well- developed roots were then removed from culture flasks and the roots gently washed under running tap water. They were then transferred to polybags containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags and maintained in high-density double check agronet open shade house. The plantlets were suitably irrigated with tap water .The polythene bags were periodically withdrawn to acclimatize the plantlets and when new leaves started emerging the polybags were completely removed. Hardened plantlets were subsequently transferred to soil in pots for further growth and development.

Statistical Analysis

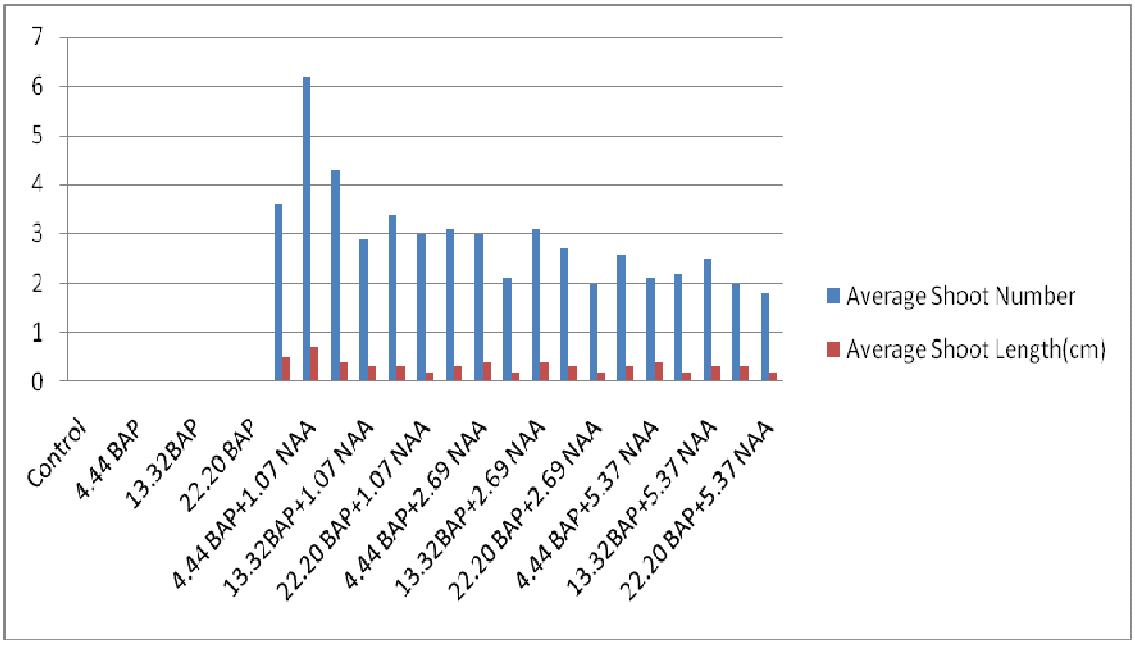
Experiments were done in triplicate and each treatment consisted of minimum ten replicates. The data was analysed using analysis of variance (ANOVA) of Completely randomized design (CRD) in GenStat Discovery Edition 13.The significance level was determined at P < = 0.05.Mean values of treatments were compared with Least Significance Difference (LSD).

Results:

***In vitro* shoot organogenesis**

De novo shoot organogenesis occurred only on proximal or middle sections of the roots. Distal regions of root explants proved to be incompetent for the purpose. Among the different treatments tried for shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) was found to be most optimal giving an average of 6.7 shoots per explant with mean shoot length of 0.7 cm without any growth of lateral roots within a period of 4 weeks [Figure 1]. MS medium without any plant growth regulator or augmented with BAP alone proved to be ineffective for shoot regeneration. On increasing the concentration of NAA to 2.69 µM and 5.37 µM , an enhanced induction of lateral roots was observed with a marked decrease in shoot bud induction from explants.(Graph 1).

Graph 1: Effect of PGR (µM) Treatment on Adventitious Shoot Regeneration from Root Explants



***In vitro* shoot multiplication**

The media combinations reported to be most optimal in our previous study on *S.chirata* (Pant *et al*., 2010) were MS medium supplemented with different concentrations of BAP, IAA and /or Ads for shoot multiplication and half-strength MS containing IBA for subsequent rooting. Similar combinations were tried in the present study and favourable results were obtained. On transferring *in vitro* grown shoots onto multiplication media, all media combinations tested were capable of inducing multiplication within 2-3 wks. Final observations in terms of mean number of shoots and shoot length were recorded after a period of 4 weeks and 8 weeks. All the treatments were significantly superior to the control except the treatments C4 and C6. In C4 (MS + 13.32 µM BAP) the average shoot number after 4 weeks and in C6 [MS+ 13.32 µM BAP+ 2.85 µM IAA] the average shoot length after 8 weeks was at par with the control. Amongst all, treatment C8 was found to be the best in all parameters. In this combination(MS + 4.44 µM BAP + 2.85 µM IAA + 271.45µM Ads) an average of 10.0 number of shoots with shoot length 2.0 cm after 4 weeks and mean shoot number 18.7 with shoot length 4.0 cm after 8 weeks was recorded [Table1] [Figure 3] .

Table 1: Effect of Plant Growth Regulators on *In vitro* Multiplication of Regenerated Microshoots

Treatment	MS medium +PGR(µM)			Average number of shoots		Average length of shoots(cm)	
	BAP	IAA	Ads	After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
C1	0.0	0.0	0.0	3.5	4	0.7	1.0
C2	4.44	0.0	0.0	4.4	7.4	1.6	2.3
C3	8.88	0.0	0.0	5.4	15	2.3	3.3
C4	13.32	0.0	0.0	5.3	9.1	1.2	1.7
C5	4.44	2.85	0.0	7.3	10.7	1.2	1.8
C6	8.88	2.85	0.0	6.4	9.8	1.1	1.6
C7	13.32	2.85	0.0	6.3	12.4	1.3	2.4
C8	**4.44**	**2.85**	**271.45**	**10.0**	**18.7**	**2.0**	**4.0**
C9	8.88	2.85	271.45	7.4	13.4	1.7	4.6
C10	13.32	2.85	271.45	8.2	11.5	1.9	2.7
Mean				6.4	11.2	1.5	2.5
LSD				1.8	2.8	0.4	1.8

After standardizing the media combination for shoot multiplication, *in vitro* multiplied shoots were subcultured on MS medium fortified with 4.44 µM BAP for maintenance of cultures.

***In vitro* rooting**

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama 1970). For the purpose, regenerated shoots were inoculated onto rooting media. It was observed that root primordia emerged from the shoot base within 10 to 14 days after shoot inoculation in all the treatments. Observations regarding mean root number and root length were recorded after 4 weeks and 8 weeks. All treatments were significantly superior to control. Optimal response was observed in the treatment R4(4.90 µM IBA) with average number of 21.0 roots per shoot after 4-weeks, 28.9 after 10-weeks and average root length 0.9 cm after 4-weeks & 1.6 cm after 10-weeks was observed in [Table 2] [Figure 4].

Table 2: Effect of Different Concentrations of IBA on Rooting of *In-vitro* Regenerated Shoots.

Treatment	1/2 strength MS medium +IBA (µM)	Average number of roots		Average length of roots(cm)	
		After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
R1	0.0	1.6	1.8	0.2	0.2
R2	0.98	6.2	12.1	0.6	1.1
R3	2.46	7.1	13.5	0.8	1.3
R4	**4.90**	**21.0**	**28.9**	**0.9**	**1.6**
R5	9.80	14.0	20.7	0.6	1.1
Mean		10.0	15.4	0.6	1.1
LSD		5.4	6.2	0.3	0.3

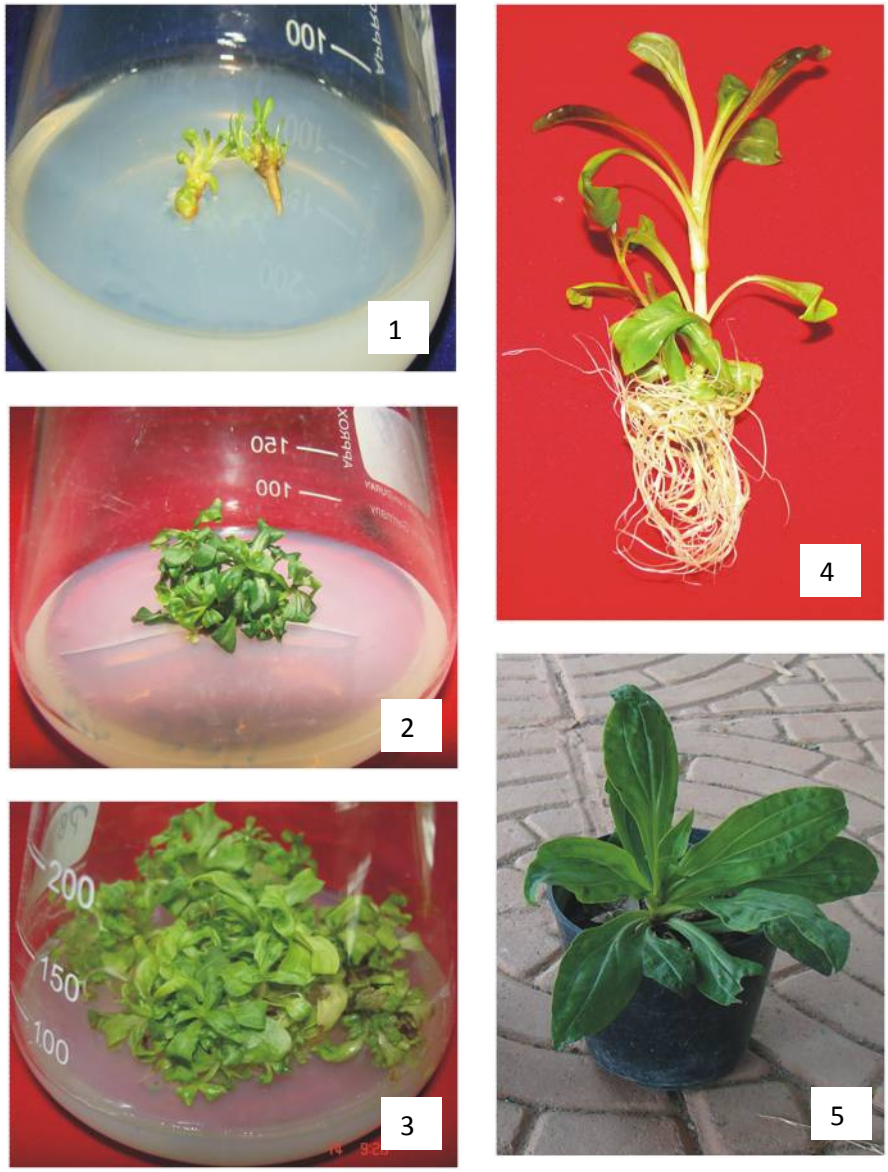


Fig. 1-5 Direct organogenesis of *Swertia chirata* via root explant:
(1) Shoot induction on root segments (2) Culture establishment
(3) Multiplication of shoots (4) *In vitro* rooted plantlet (5) Hardened plantlet.

Hardening and acclimatization

In-vitro hardening was done on liquid 1/4MS medium having 2% sucrose, for 7 days followed by acclimatization in agronet shade house in polybags containing a mixture of soil: sand :manure (1:1:1) and covered with perforated polythene bags. These polythene bags were withdrawn periodically and on emergence of new leaves, the polybags were completely removed. The plants maintained in shade house were irrigated with water. For next one month,

plants were maintained in net house. Well-acclimatized plants were shifted to pots containing soil and gave percentage survival of over 80%. [Figure 5].

Discussion

Careful observations revealed that of the different root segments tried for shoot bud initiation in our study, distal root segments remained non-responsive to shoot bud initiation while proximal and middle segments exhibited shoot regeneration and apparently no difference in the frequency and mode of shoot bud differentiation. Bud initiation and its formation uniformly from the proximal or middle portion of the root indicate that some secondary growth may also be essential for shoot initiation. The results are in corroboration with reports on other species where only the proximal and middle root sections exhibited *de novo* shoot organogenesis in *Comptonia peregrine* (Goforth and Torrey,1977), *Brassica napus* (Sharma and Thorpe, 1987), *Holostemma annulare* (Sudha *et al.*, 2000), *Populus tremula* (Vinocur *et al*.,2000), *Hypericum perforatum* (Zobayed and Saxena, 2003).

In the present study, among the different treatments tried for *in vitro* shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) gave maximum number of shoots without any growth of lateral roots within a period of 3 weeks . A synergistic effect of BAP and NAA has been highlighted in previous studies on shoot initiation from root explants of *Dalbergia sissoo* (Mukhopadhay and Mohan Ram,1981); *Citrus sinensis* (Burger and Hackett, 1986); *Citrus mitis* (Sim *et al.*, 1989); *Averrhoa carambola* (Kantharajah *et al.*,1992); *Clitoria ternatea* (Shahzad *et al.,*2007) and *Podophyllum hexandrum* (Chakraborty *et al*. ,2010) .

However, in our study, on increasing the concentration of NAA to 2.69 µM and 5.37 µM, significant reduction in shoot bud initiation was observed with an enhanced induction of lateral roots from cut end of roots. Root explants in our study were taken from *in vitro* regenerated roots growing in auxin-rich medium. On placing the explants in another auxin- rich medium may be an inhibiting factor for shoot bud differentiation while facilitating lateral root formation. This is also supported by the view that relatively high concentration of auxin suppresses the inception of shoot buds and promotes growth of lateral roots (Peterson, 1975). Cytokinins are considered to stimulate bud formation and inhibit lateral root formation. There are reports where BAP alone has found to be most efficient for shoot bud regeneration from root tissues in *Citrus mitis* (Sim *et al.,* 1989); *Citrus aurantifolia* (Bhat *et al.*, 1992); *Holostemma annulare* (Sudha *et al*., 2000); *Garnicia indica* (Deodhar *et al.*, 2000); *Blackstonia perfoliata* (Bijelovic *et al*. ,2004); *Crataeva nurvula* (Walia *et al.*,2004); *Melia azedarach* (Vila *et al.*, 2005). Contrastingly, in our study BAP when tried alone at different concentrations was found to be completely inefficient for shoot regeneration.

Wawrosch *et al.,* 1999 reported adventitious shoot regeneration from root explants of *S. chirata* where radicle explants from 3-week-old aseptically grown seedlings were used as primary explants for shoot organogenesis. They optimized a procedure of 3 weeks culturing root explants on BAP(3µM) supplemented MS medium followed by transfer to basal MS medium for another 3 weeks. However, the regeneration procedure described was not very efficient as only 1.9 shoots per explant could be achieved and the regenerated shoots were hyperhydrated. We report a significant improvement over these results as an average of 6.7 shoots per root explants could be obtained in our study which were further multiplied on MS medium supplemented with BAP, IAA and Ads and 18.0 fold multiplication was achieved. The regenerated shoots were healthy and robust without any observation of hyperhydration.

Rooting experiments were initiated after 180 days of subculturing to get maximum material for trying different hormone concentrations. Half-strength MS medium supplemented with IBA at 4.90 µM concentration proved to be most optimal for rooting. The results were similar to our previous findings on *in vitro* propagation of *S. chirata* via nodal explants. Wawrosch *et al.*(1999) had reported callus formation on exposure of shoots to media supplemented with upto 10 µM auxins for rooting. Therefore, they described a method of 2second dipping of shoots in an aqueous solution of 15 ppm NAA followed by 3-4 week cultivation period on hormone-free, half-strength MS medium for root development. In contrast, callusing did not occur in any of the treatments tried for *in vitro* rooting in our study and long, healthy root system developed on IBA augmented medium.

In vitro raised plants are heterotrophic in their mode of nutrition and cannot withstand the environmental conditions without proper hardening and acclimatization, thus, need to be hardened and acclimatized before field transplantation. Regenerated plantlets were hardened under controlled conditions of culture room on ¼ liquid MS medium and sucrose supply was decreased to 2%. After a period of one week, these plantlets were transferred to a rooting mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags in shade. Holes were made in polythene bags for gaseous

exchange. For acclimatizing the plantlets, bags were withdrawn periodically. During the process, the shoots elongated, leaves turned greener and expanded after which the polybags were completely removed. Hardened plants were subsequently transferred to soil filled in pots and over 80 % survivability was recorded.

We suggest that this ability of *S.chirata* plants of direct shoot organogenesis via root in nature, juvenile tissues of the root and specific combinations of growth regulators collectively triggered the process of direct shoot differentiation from root explants *in vitro*.

Conclusion

This study has revealed the morphogenetic potential of *in vitro* grown roots of *S.chirata* as a source tissue for micropropagation. The explants can be easily and regularly obtained from established shoot cultures and do not require disinfection treatment hence being ideal for germplasm exchange and cryopreservation. Normal root culture establishment for a number of plant species have the ability to accumulate secondary products similar to those found in mother donor plant (Tabata *et al.*, 1972; Whitten *et al.,* 1981). In *S.chirata* root being the most important part of the plant for medicinal purposes holds importance as the starting material for tissue culture studies. The present protocol will therefore serve as alternative means for *in vitro* clonal propagation of this endangered plant with immense medicinal value and further biochemical and physiological investigations.

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De novo Shoot Organogenesis from Cultured Root Explants of *Swertia chirata* Buch.-Ham.ex Wall.: An Endangered Medicinal Plant.

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Abstract: *In vitro* regeneration of plants from root cultures of *Swertia chirata* Buch.-Ham. ex Wall was obtained. Root explants from axenic shoot cultures were used for shoot induction. Optimal shoot regeneration without any callus intervention was observed on 1/2 MS (Murashige and Skoog's medium, 1962) + 4.44 µM 6, benzylaminopurine (BAP) +1.07 µM α-naphthalene acetic acid (NAA).Regenerated shoots were further multiplied on full strength MS medium supplemented with different concentrations of plant growth regulators. Maximum shoot multiplication was achieved on MS medium fortified with 4.44 µM BAP + 2.85 µM indole-3 acetic acid (IAA) + 271.45 µM adenine sulphate (Ads). Best results of rooting were observed on 1/2 strength MS medium containing 4.90 µM IBA. Plants with well-developed shoots and roots were successfully hardened with over 80% survivability.
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Keywords: *Swertia chirata*, root culture, *de novo* organogenesis, *in vitro* plant regeneration.

Abbreviations: MS: Murashige and Skoog (1962); **BAP:** 6, Benzylaminopurine; **Kn:** 6-Furfurylaminopurine; **NAA:** α-Naphthalene Acetic Acid; **IBA:** Indole-3 butyric acid; **IAA**: Indole-3 acetic acid; **GA_3**: Gibberellic acid; **Ads:** Adenine sulphate; **PGR:** Plant growth regulator

Introduction

Swertia chirata Buch.- Ham.- commonly known as chirayata is a medicinal plant of family Gentianaceae is native to temperate Himalaya (altitude above 4,000-10,000 ft.). The plant has an erect, about 2-3 ft long stem and the whole plant is bitter in taste. *S.chirata* contains a yellow bitter ophelic acid and two bitter glucosides chiratin and amarogentin. The root is said to be the most powerful part which is included in the British and American Pharmacopoeias and is used against a variety of diseases. The bitterness, antihelmintic, hypoglycemic and antipyretic properties are attributed to amarogentin ; most bitter compound isolated till date (Karan et al., 1996), swerchirin, swertiamarin and other active principles of the herb. The whole plant contains gentiamine alkaloids and the aerial part contains xanthones. Herbal medicines such as Ayush-64, Diabecon, Mensturyl syrup and Melicon ointment contain chirata extract in different amounts (Edwin et al., 1988, Valecha et al., 2000 and Mitra et al., 1996). The drug obtained from the dried plant is held in high esteem in India and is prescribed in a variety of forms and combinations in chronic fever, anemia and is used as special remedy for bronchial asthma, liver and stomach disorders, malaria and diabetes.

Several species of the genus *Swertia* are available in India and other countries and used as adulterant. However, *S.chirata* from India *and S.japonica* and *S.pseudochinenesis* from Japan are considered elite species because of their medicinal value and revenue-generating potential.

Chirayata is difficult to propagate on mass scale via seed owing to non-availability of seeds or due to harvesting of plants before seeds mature. The species is, therefore, deprived of natural regeneration. Also, the low viability and germination percentage of seeds and the necessary delicate field handling of the seedlings are some of the factors that discourage agro technology development and commercial cultivation of the species. There is an increasing pharmaceutical demand of the species both, in the indigenous and the world market but lack of large-scale commercial plantations of the herb continues. Destructive harvesting and loss of habitat has diminished the existing populations of *S.chirata*. The species is among the highly prioritized medicinal plants of India as identified by National Medicinal Plant Board, Govt. Of India and according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria, this priority plant has been designated as critically endangered .The need to develop techniques for its mass multiplication through different regeneration pathways thus, becomes imperative.

Documented literature reveals that there are limited reports on *in vitro* propagation of *Swertia chirata.* Micropropagation via field-grown nodal explants has been reported by Ahuja *et al.* (2003), Chaudhuri *et al.* (2007), Koul *et al*. (2009) and Pant *et al.,* 2010. Joshi and Dhawan (2007 a) and Balaraju *et al.* (2009) published reports on *in vitro* propagation of *S. chirata* using shoot tip explants derived from *in vitro* grown seedlings. ISSR marker analysis of genetic diversity among *S.chirata* genotypes of temperate Himalayan region of India was done by Joshi and Dhawan (2007 b). Chaudhuri *et al.* 2008 and 2009 reported direct shoot regeneration from *in vitro* leaves regeneration via immature seed cultures of *S. chirata*. Wang *et al.* (2009) described *in vitro* shoot regeneration from leaves taken from field-grown plants.

Root tissue has been proven to be highly regenerative explant and is relatively easy to maintain and manipulate *in vitro* (Vincour *et al.*, 2000; Franklin, 2004). Besides, shoots developing on root segments of the same plant have been reported to be genetically uniform and the method, an alternative way for clonal propagation. (Ostazeski and Henson, 1965; Budd, 1973; Chaturvedi *et al.*, 1981; Sharma *et al.*, 1993). The only available report on culture of root segments of *S.chirata* procured from *in vitro* raised seedlings was by Wawrosch *et al.* (1999). However, low frequency of shoot regeneration and hyperhydration of shoots hindered the production of quality shoots employing the procedure described by them. The present report communicates a reproducible protocol for regeneration of well-developed and healthy *S. chirata* plantlets via root segment culture without any intervening callus phase. The findings will be useful in conservation and mass multiplication of this endangered medicinal plant.

Material and Methods

Explant Source

In our previous study on *S.chirata* (Pant *et al.,* 2010) the shoots were induced from nodal explants. Regenerated shoots were further multiplied , subcultured and maintained on full strength MS medium supplemented with BAP(4.44 µM). Shoots having 1 or 2 nodes were rooted on half-strength MS medium supplemented with 4.90 µM indole-3 butyric acid (IBA).Ten – weeks old *in vitro* developed roots were used as an explant material for direct shoot organogenesis. For this, *in vitro* regenerated shoots via nodal explants were rooted on half-strength MS medium supplemented with 2% sucrose and IBA (4.90 µM). Aseptically removed long roots were repeatedly washed in sterile distilled water to free them from agar adhering to the surface. Subsequently they were dissected into 2-3 cm long proximal, middle and distal segments and placed horizontally on the culture medium for shoot organogenesis.

In vitro Shoot Organogenesis

Half-strength MS medium solidified with 0.7% agar (w/v) and supplemented with 2% sucrose and varying concentrations and combinations of phytohormones viz BAP(2.22 µM-22.20 µM) and NAA(1.07 µM- 5.37 µM) was tested for direct shoot regeneration from axenic root cultures. MS medium lacking plant growth regulators served as control. Data on average shoot number per explant and average shoot length (cm) was recorded after an interval of 4 weeks.

In vitro Shoot Multiplication

Once the culture conditions for optimum shoot induction from root explants were established, the proliferated shoots were subcultured onto fresh medium of same composition to establish an initial stock of shoots. These shoots were further used to assess the effect of growth regulators on further *in vitro* shoot multiplication. Full strength MS medium agarified with 0.7% agar (w/v) and fortified with 3% sucrose and varying concentrations and combinations of plant growth regulators were used for the purpose. Experiments conducted to obtain maximum shoot multiplication largely consisted of experiments pertaining to cytokinin (4.44 µM – 13.32 µM BAP) alone and in combination with auxin (1.14 µM – 2.85 µM IAA) and/or adjuvant adenine sulphate (271.45 µM). Observations pertaining to average shoot number and average shoot length were recorded after periodic intervals of 4 weeks and 8 weeks. Routine sub culturing of multiple shoots was carried out at an interval of every 3-week.

In vitro Rooting

Experiments on *in vitro* rooting of *in vitro* grown shoots were attempted with 2-3 cm long shoots produced during shoot multiplication. Shoots were cultured in each tube on MS medium containing 2% sucrose and supplemented with auxins IAA (1.14 µM - 11.40 µM), IBA (0.98 µM - 9.80 µM) and NAA (1.07 µM - 10.74 µM). Single shoots were cultured for initiation of roots and observations were recorded after 4 weeks and 8 weeks. Rooting response was recorded in terms of average number of roots produced and average root length.

Medium and Culture conditions

The pH of medium was adjusted to 5.8 before adding 0.7% agar (w/v) and sterilized by autoclaving at 15lbs (1.8 kg/cm^2) pressure at 121^{0}C for 15 minutes. Cultures were incubated at 25 ± 1^{0}C

for 16 hours in light (illuminated by 40 watt fluorescent tubes, 50µE m^{-2} s^{-1}) and for 8 hours in dark cycle irradiance by cool fluorescent tubes.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to liquid 1/4 strength MS strength medium having 2% sucrose and devoid of any plant growth regulator, for 7 days in flasks. Plantlets with well- developed roots were then removed from culture flasks and the roots gently washed under running tap water. They were then transferred to polybags containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags and maintained in high-density double check agronet open shade house. The plantlets were suitably irrigated with tap water .The polythene bags were periodically withdrawn to acclimatize the plantlets and when new leaves started emerging the polybags were completely removed. Hardened plantlets were subsequently transferred to soil in pots for further growth and development.

Statistical Analysis

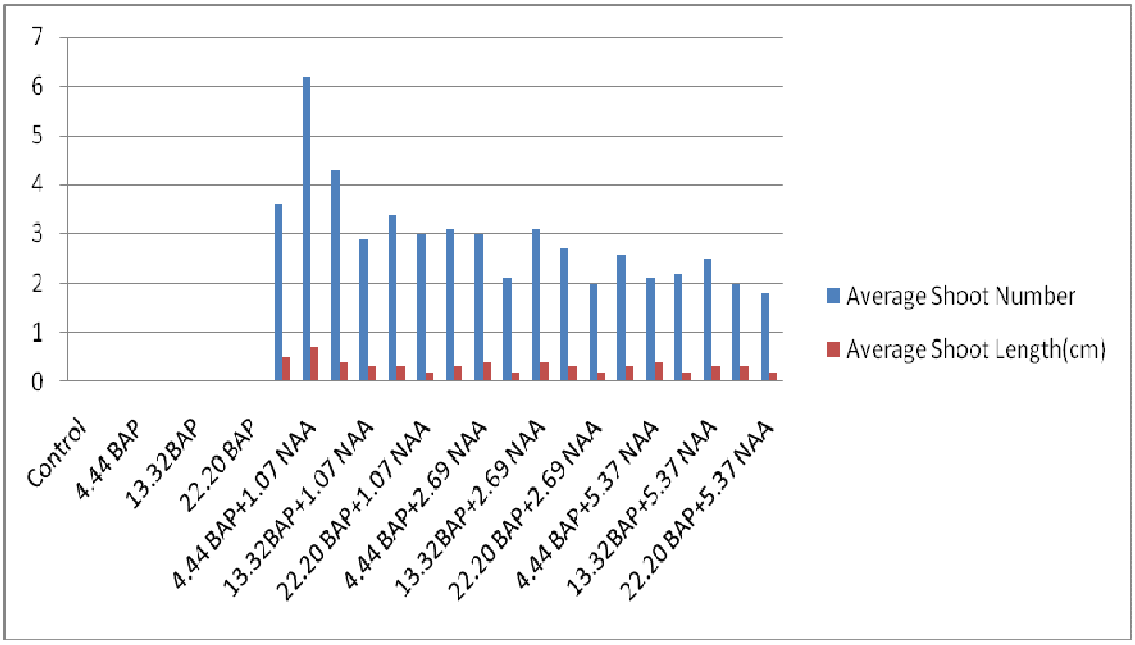
Experiments were done in triplicate and each treatment consisted of minimum ten replicates. The data was analysed using analysis of variance (ANOVA) of Completely randomized design (CRD) in GenStat Discovery Edition 13.The significance level was determined at P < = 0.05.Mean values of treatments were compared with Least Significance Difference (LSD).

Results:

***In vitro* shoot organogenesis**

De novo shoot organogenesis occurred only on proximal or middle sections of the roots. Distal regions of root explants proved to be incompetent for the purpose. Among the different treatments tried for shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) was found to be most optimal giving an average of 6.7 shoots per explant with mean shoot length of 0.7 cm without any growth of lateral roots within a period of 4 weeks [Figure 1]. MS medium without any plant growth regulator or augmented with BAP alone proved to be ineffective for shoot regeneration. On increasing the concentration of NAA to 2.69 µM and 5.37 µM , an enhanced induction of lateral roots was observed with a marked decrease in shoot bud induction from explants.(Graph 1).

Graph 1: Effect of PGR (µM) Treatment on Adventitious Shoot Regeneration from Root Explants



***In vitro* shoot multiplication**

The media combinations reported to be most optimal in our previous study on *S.chirata* (Pant *et al*., 2010) were MS medium supplemented with different concentrations of BAP, IAA and /or Ads for shoot multiplication and half-strength MS containing IBA for subsequent rooting. Similar combinations were tried in the present study and favourable results were obtained. On transferring *in vitro* grown shoots onto multiplication media, all media combinations tested were capable of inducing multiplication within 2-3 wks. Final observations in terms of mean number of shoots and shoot length were recorded after a period of 4 weeks and 8 weeks. All the treatments were significantly superior to the control except the treatments C4 and C6. In C4 (MS + 13.32 µM BAP) the average shoot number after 4 weeks and in C6 [MS+ 13.32 µM BAP+ 2.85 µM IAA] the average shoot length after 8 weeks was at par with the control. Amongst all, treatment C8 was found to be the best in all parameters. In this combination(MS + 4.44 µM BAP + 2.85 µM IAA + 271.45µM Ads) an average of 10.0 number of shoots with shoot length 2.0 cm after 4 weeks and mean shoot number 18.7 with shoot length 4.0 cm after 8 weeks was recorded [Table1] [Figure 3] .

Table 1: Effect of Plant Growth Regulators on *In vitro* Multiplication of Regenerated Microshoots

Treatment	MS medium +PGR(µM)			Average number of shoots		Average length of shoots(cm)	
	BAP	IAA	Ads	After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
C1	0.0	0.0	0.0	3.5	4	0.7	1.0
C2	4.44	0.0	0.0	4.4	7.4	1.6	2.3
C3	8.88	0.0	0.0	5.4	15	2.3	3.3
C4	13.32	0.0	0.0	5.3	9.1	1.2	1.7
C5	4.44	2.85	0.0	7.3	10.7	1.2	1.8
C6	8.88	2.85	0.0	6.4	9.8	1.1	1.6
C7	13.32	2.85	0.0	6.3	12.4	1.3	2.4
C8	**4.44**	**2.85**	**271.45**	**10.0**	**18.7**	**2.0**	**4.0**
C9	8.88	2.85	271.45	7.4	13.4	1.7	4.6
C10	13.32	2.85	271.45	8.2	11.5	1.9	2.7
Mean				6.4	11.2	1.5	2.5
LSD				1.8	2.8	0.4	1.8

After standardizing the media combination for shoot multiplication, *in vitro* multiplied shoots were subcultured on MS medium fortified with 4.44 µM BAP for maintenance of cultures.

***In vitro* rooting**

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama 1970). For the purpose, regenerated shoots were inoculated onto rooting media. It was observed that root primordia emerged from the shoot base within 10 to 14 days after shoot inoculation in all the treatments. Observations regarding mean root number and root length were recorded after 4 weeks and 8 weeks. All treatments were significantly superior to control. Optimal response was observed in the treatment R4(4.90 µM IBA) with average number of 21.0 roots per shoot after 4-weeks, 28.9 after 10-weeks and average root length 0.9 cm after 4-weeks & 1.6 cm after 10-weeks was observed in [Table 2] [Figure 4].

Table 2: Effect of Different Concentrations of IBA on Rooting of *In-vitro* Regenerated Shoots.

Treatment	1/2 strength MS medium +IBA (µM)	Average number of roots		Average length of roots(cm)	
		After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
R1	0.0	1.6	1.8	0.2	0.2
R2	0.98	6.2	12.1	0.6	1.1
R3	2.46	7.1	13.5	0.8	1.3
R4	**4.90**	**21.0**	**28.9**	**0.9**	**1.6**
R5	9.80	14.0	20.7	0.6	1.1
Mean		10.0	15.4	0.6	1.1
LSD		5.4	6.2	0.3	0.3

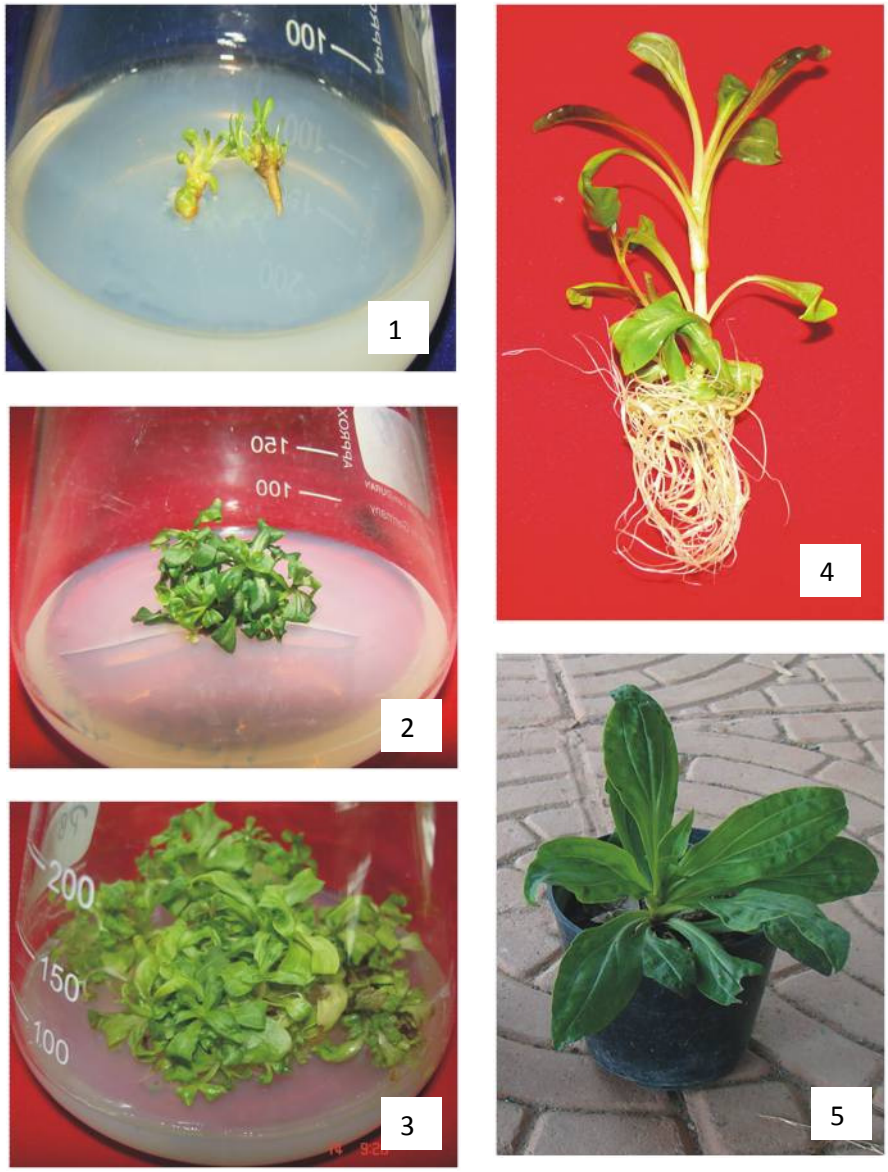


Fig. 1-5 Direct organogenesis of *Swertia chirata* via root explant:
(1) Shoot induction on root segments (2) Culture establishment
(3) Multiplication of shoots (4) *In vitro* rooted plantlet (5) Hardened plantlet.

Hardening and acclimatization

In-vitro hardening was done on liquid 1/4MS medium having 2% sucrose, for 7 days followed by acclimatization in agronet shade house in polybags containing a mixture of soil: sand :manure (1:1:1) and covered with perforated polythene bags. These polythene bags were withdrawn periodically and on emergence of new leaves, the polybags were completely removed. The plants maintained in shade house were irrigated with water. For next one month,

plants were maintained in net house. Well-acclimatized plants were shifted to pots containing soil and gave percentage survival of over 80%. [Figure 5].

Discussion

Careful observations revealed that of the different root segments tried for shoot bud initiation in our study, distal root segments remained non-responsive to shoot bud initiation while proximal and middle segments exhibited shoot regeneration and apparently no difference in the frequency and mode of shoot bud differentiation. Bud initiation and its formation uniformly from the proximal or middle portion of the root indicate that some secondary growth may also be essential for shoot initiation. The results are in corroboration with reports on other species where only the proximal and middle root sections exhibited *de novo* shoot organogenesis in *Comptonia peregrine* (Goforth and Torrey,1977), *Brassica napus* (Sharma and Thorpe, 1987), *Holostemma annulare* (Sudha *et al.*, 2000), *Populus tremula* (Vinocur *et al*.,2000), *Hypericum perforatum* (Zobayed and Saxena, 2003).

In the present study, among the different treatments tried for *in vitro* shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) gave maximum number of shoots without any growth of lateral roots within a period of 3 weeks . A synergistic effect of BAP and NAA has been highlighted in previous studies on shoot initiation from root explants of *Dalbergia sissoo* (Mukhopadhay and Mohan Ram,1981); *Citrus sinensis* (Burger and Hackett, 1986); *Citrus mitis* (Sim *et al.*, 1989); *Averrhoa carambola* (Kantharajah *et al.*,1992); *Clitoria ternatea* (Shahzad *et al.,*2007) and *Podophyllum hexandrum* (Chakraborty *et al*. ,2010) .

However, in our study, on increasing the concentration of NAA to 2.69 µM and 5.37 µM, significant reduction in shoot bud initiation was observed with an enhanced induction of lateral roots from cut end of roots. Root explants in our study were taken from *in vitro* regenerated roots growing in auxin-rich medium. On placing the explants in another auxin- rich medium may be an inhibiting factor for shoot bud differentiation while facilitating lateral root formation. This is also supported by the view that relatively high concentration of auxin suppresses the inception of shoot buds and promotes growth of lateral roots (Peterson, 1975). Cytokinins are considered to stimulate bud formation and inhibit lateral root formation. There are reports where BAP alone has found to be most efficient for shoot bud regeneration from root tissues in *Citrus mitis* (Sim *et al.,* 1989); *Citrus aurantifolia* (Bhat *et al.*, 1992); *Holostemma annulare* (Sudha *et al*., 2000); *Garnicia indica* (Deodhar *et al.*, 2000); *Blackstonia perfoliata* (Bijelovic *et al*. ,2004); *Crataeva nurvula* (Walia *et al.*,2004); *Melia azedarach* (Vila *et al.*, 2005). Contrastingly, in our study BAP when tried alone at different concentrations was found to be completely inefficient for shoot regeneration.

Wawrosch *et al.,* 1999 reported adventitious shoot regeneration from root explants of *S. chirata* where radicle explants from 3-week-old aseptically grown seedlings were used as primary explants for shoot organogenesis. They optimized a procedure of 3 weeks culturing root explants on BAP(3µM) supplemented MS medium followed by transfer to basal MS medium for another 3 weeks. However, the regeneration procedure described was not very efficient as only 1.9 shoots per explant could be achieved and the regenerated shoots were hyperhydrated. We report a significant improvement over these results as an average of 6.7 shoots per root explants could be obtained in our study which were further multiplied on MS medium supplemented with BAP, IAA and Ads and 18.0 fold multiplication was achieved. The regenerated shoots were healthy and robust without any observation of hyperhydration.

Rooting experiments were initiated after 180 days of subculturing to get maximum material for trying different hormone concentrations. Half-strength MS medium supplemented with IBA at 4.90 µM concentration proved to be most optimal for rooting. The results were similar to our previous findings on *in vitro* propagation of *S. chirata* via nodal explants. Wawrosch *et al.*(1999) had reported callus formation on exposure of shoots to media supplemented with upto 10 µM auxins for rooting. Therefore, they described a method of 2second dipping of shoots in an aqueous solution of 15 ppm NAA followed by 3-4 week cultivation period on hormone-free, half-strength MS medium for root development. In contrast, callusing did not occur in any of the treatments tried for *in vitro* rooting in our study and long, healthy root system developed on IBA augmented medium.

In vitro raised plants are heterotrophic in their mode of nutrition and cannot withstand the environmental conditions without proper hardening and acclimatization, thus, need to be hardened and acclimatized before field transplantation. Regenerated plantlets were hardened under controlled conditions of culture room on ¼ liquid MS medium and sucrose supply was decreased to 2%. After a period of one week, these plantlets were transferred to a rooting mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags in shade. Holes were made in polythene bags for gaseous

exchange. For acclimatizing the plantlets, bags were withdrawn periodically. During the process, the shoots elongated, leaves turned greener and expanded after which the polybags were completely removed. Hardened plants were subsequently transferred to soil filled in pots and over 80 % survivability was recorded.

We suggest that this ability of *S.chirata* plants of direct shoot organogenesis via root in nature, juvenile tissues of the root and specific combinations of growth regulators collectively triggered the process of direct shoot differentiation from root explants *in vitro*.

Conclusion

This study has revealed the morphogenetic potential of *in vitro* grown roots of *S.chirata* as a source tissue for micropropagation. The explants can be easily and regularly obtained from established shoot cultures and do not require disinfection treatment hence being ideal for germplasm exchange and cryopreservation. Normal root culture establishment for a number of plant species have the ability to accumulate secondary products similar to those found in mother donor plant (Tabata *et al.*, 1972; Whitten *et al.,* 1981). In *S.chirata* root being the most important part of the plant for medicinal purposes holds importance as the starting material for tissue culture studies. The present protocol will therefore serve as alternative means for *in vitro* clonal propagation of this endangered plant with immense medicinal value and further biochemical and physiological investigations.

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De novo Shoot Organogenesis from Cultured Root Explants of *Swertia chirata* Buch.-Ham.ex Wall.: An Endangered Medicinal Plant.

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Abstract: *In vitro* regeneration of plants from root cultures of *Swertia chirata* Buch.-Ham. ex Wall was obtained. Root explants from axenic shoot cultures were used for shoot induction. Optimal shoot regeneration without any callus intervention was observed on 1/2 MS (Murashige and Skoog's medium, 1962) + 4.44 µM 6, benzylaminopurine (BAP) +1.07 µM α-naphthalene acetic acid (NAA).Regenerated shoots were further multiplied on full strength MS medium supplemented with different concentrations of plant growth regulators. Maximum shoot multiplication was achieved on MS medium fortified with 4.44 µM BAP + 2.85 µM indole-3 acetic acid (IAA) + 271.45 µM adenine sulphate (Ads). Best results of rooting were observed on 1/2 strength MS medium containing 4.90 µM IBA. Plants with well-developed shoots and roots were successfully hardened with over 80% survivability.
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Keywords: *Swertia chirata*, root culture, *de novo* organogenesis, *in vitro* plant regeneration.

Abbreviations: MS: Murashige and Skoog (1962); **BAP:** 6, Benzylaminopurine; **Kn:** 6-Furfurylaminopurine; **NAA:** α-Naphthalene Acetic Acid; **IBA:** Indole-3 butyric acid; **IAA**: Indole-3 acetic acid; **GA_3**: Gibberellic acid; **Ads:** Adenine sulphate; **PGR:** Plant growth regulator

Introduction

Swertia chirata Buch.- Ham.- commonly known as chirayata is a medicinal plant of family Gentianaceae is native to temperate Himalaya (altitude above 4,000-10,000 ft.). The plant has an erect, about 2-3 ft long stem and the whole plant is bitter in taste. *S.chirata* contains a yellow bitter ophelic acid and two bitter glucosides chiratin and amarogentin. The root is said to be the most powerful part which is included in the British and American Pharmacopoeias and is used against a variety of diseases. The bitterness, antihelmintic, hypoglycemic and antipyretic properties are attributed to amarogentin ; most bitter compound isolated till date (Karan et al., 1996), swerchirin, swertiamarin and other active principles of the herb. The whole plant contains gentiamine alkaloids and the aerial part contains xanthones. Herbal medicines such as Ayush-64, Diabecon, Mensturyl syrup and Melicon ointment contain chirata extract in different amounts (Edwin et al., 1988, Valecha et al., 2000 and Mitra et al., 1996). The drug obtained from the dried plant is held in high esteem in India and is prescribed in a variety of forms and combinations in chronic fever, anemia and is used as special remedy for bronchial asthma, liver and stomach disorders, malaria and diabetes.

Several species of the genus *Swertia* are available in India and other countries and used as adulterant. However, *S.chirata* from India *and S.japonica* and *S.pseudochinenesis* from Japan are considered elite species because of their medicinal value and revenue-generating potential.

Chirayata is difficult to propagate on mass scale via seed owing to non-availability of seeds or due to harvesting of plants before seeds mature. The species is, therefore, deprived of natural regeneration. Also, the low viability and germination percentage of seeds and the necessary delicate field handling of the seedlings are some of the factors that discourage agro technology development and commercial cultivation of the species. There is an increasing pharmaceutical demand of the species both, in the indigenous and the world market but lack of large-scale commercial plantations of the herb continues. Destructive harvesting and loss of habitat has diminished the existing populations of *S.chirata*. The species is among the highly prioritized medicinal plants of India as identified by National Medicinal Plant Board, Govt. Of India and according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria, this priority plant has been designated as critically endangered .The need to develop techniques for its mass multiplication through different regeneration pathways thus, becomes imperative.

Documented literature reveals that there are limited reports on *in vitro* propagation of *Swertia chirata.* Micropropagation via field-grown nodal explants has been reported by Ahuja *et al.* (2003), Chaudhuri *et al.* (2007), Koul *et al*. (2009) and Pant *et al.,* 2010. Joshi and Dhawan (2007 a) and Balaraju *et al.* (2009) published reports on *in vitro* propagation of *S. chirata* using shoot tip explants derived from *in vitro* grown seedlings. ISSR marker analysis of genetic diversity among *S.chirata* genotypes of temperate Himalayan region of India was done by Joshi and Dhawan (2007 b). Chaudhuri *et al.* 2008 and 2009 reported direct shoot regeneration from *in vitro* leaves regeneration via immature seed cultures of *S. chirata*. Wang *et al.* (2009) described *in vitro* shoot regeneration from leaves taken from field-grown plants.

Root tissue has been proven to be highly regenerative explant and is relatively easy to maintain and manipulate *in vitro* (Vincour *et al.*, 2000; Franklin, 2004). Besides, shoots developing on root segments of the same plant have been reported to be genetically uniform and the method, an alternative way for clonal propagation. (Ostazeski and Henson, 1965; Budd, 1973; Chaturvedi *et al.*, 1981; Sharma *et al.*, 1993). The only available report on culture of root segments of *S.chirata* procured from *in vitro* raised seedlings was by Wawrosch *et al.* (1999). However, low frequency of shoot regeneration and hyperhydration of shoots hindered the production of quality shoots employing the procedure described by them. The present report communicates a reproducible protocol for regeneration of well-developed and healthy *S. chirata* plantlets via root segment culture without any intervening callus phase. The findings will be useful in conservation and mass multiplication of this endangered medicinal plant.

Material and Methods

Explant Source

In our previous study on *S.chirata* (Pant *et al.,* 2010) the shoots were induced from nodal explants. Regenerated shoots were further multiplied , subcultured and maintained on full strength MS medium supplemented with BAP(4.44 µM). Shoots having 1 or 2 nodes were rooted on half-strength MS medium supplemented with 4.90 µM indole-3 butyric acid (IBA).Ten – weeks old *in vitro* developed roots were used as an explant material for direct shoot organogenesis. For this, *in vitro* regenerated shoots via nodal explants were rooted on half-strength MS medium supplemented with 2% sucrose and IBA (4.90 µM). Aseptically removed long roots were repeatedly washed in sterile distilled water to free them from agar adhering to the surface. Subsequently they were dissected into 2-3 cm long proximal, middle and distal segments and placed horizontally on the culture medium for shoot organogenesis.

In vitro Shoot Organogenesis

Half-strength MS medium solidified with 0.7% agar (w/v) and supplemented with 2% sucrose and varying concentrations and combinations of phytohormones viz BAP(2.22 µM-22.20 µM) and NAA(1.07 µM- 5.37 µM) was tested for direct shoot regeneration from axenic root cultures. MS medium lacking plant growth regulators served as control. Data on average shoot number per explant and average shoot length (cm) was recorded after an interval of 4 weeks.

In vitro Shoot Multiplication

Once the culture conditions for optimum shoot induction from root explants were established, the proliferated shoots were subcultured onto fresh medium of same composition to establish an initial stock of shoots. These shoots were further used to assess the effect of growth regulators on further *in vitro* shoot multiplication. Full strength MS medium agarified with 0.7% agar (w/v) and fortified with 3% sucrose and varying concentrations and combinations of plant growth regulators were used for the purpose. Experiments conducted to obtain maximum shoot multiplication largely consisted of experiments pertaining to cytokinin (4.44 µM – 13.32 µM BAP) alone and in combination with auxin (1.14 µM – 2.85 µM IAA) and/or adjuvant adenine sulphate (271.45 µM). Observations pertaining to average shoot number and average shoot length were recorded after periodic intervals of 4 weeks and 8 weeks. Routine sub culturing of multiple shoots was carried out at an interval of every 3-week.

In vitro Rooting

Experiments on *in vitro* rooting of *in vitro* grown shoots were attempted with 2-3 cm long shoots produced during shoot multiplication. Shoots were cultured in each tube on MS medium containing 2% sucrose and supplemented with auxins IAA (1.14 µM - 11.40 µM), IBA (0.98 µM - 9.80 µM) and NAA (1.07 µM - 10.74 µM). Single shoots were cultured for initiation of roots and observations were recorded after 4 weeks and 8 weeks. Rooting response was recorded in terms of average number of roots produced and average root length.

Medium and Culture conditions

The pH of medium was adjusted to 5.8 before adding 0.7% agar (w/v) and sterilized by autoclaving at 15lbs (1.8 kg/cm^2) pressure at 121^{0}C for 15 minutes. Cultures were incubated at 25 ± 1^{0}C

for 16 hours in light (illuminated by 40 watt fluorescent tubes, 50µE m^{-2} s^{-1}) and for 8 hours in dark cycle irradiance by cool fluorescent tubes.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to liquid 1/4 strength MS strength medium having 2% sucrose and devoid of any plant growth regulator, for 7 days in flasks. Plantlets with well- developed roots were then removed from culture flasks and the roots gently washed under running tap water. They were then transferred to polybags containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags and maintained in high-density double check agronet open shade house. The plantlets were suitably irrigated with tap water .The polythene bags were periodically withdrawn to acclimatize the plantlets and when new leaves started emerging the polybags were completely removed. Hardened plantlets were subsequently transferred to soil in pots for further growth and development.

Statistical Analysis

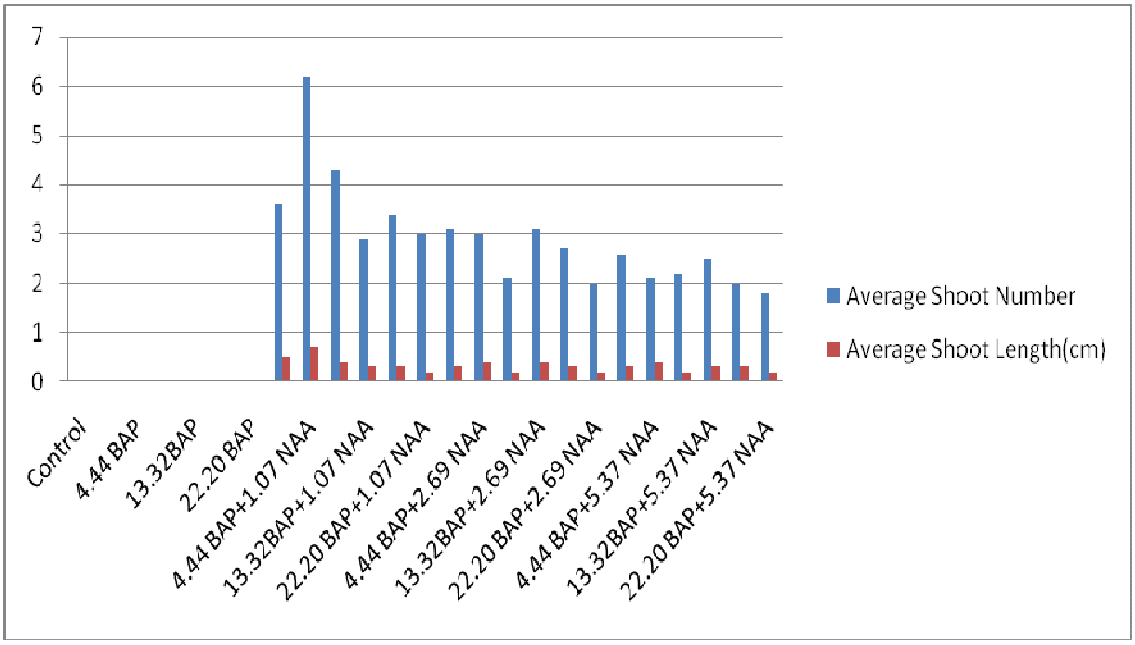
Experiments were done in triplicate and each treatment consisted of minimum ten replicates. The data was analysed using analysis of variance (ANOVA) of Completely randomized design (CRD) in GenStat Discovery Edition 13.The significance level was determined at P < = 0.05.Mean values of treatments were compared with Least Significance Difference (LSD).

Results:

***In vitro* shoot organogenesis**

De novo shoot organogenesis occurred only on proximal or middle sections of the roots. Distal regions of root explants proved to be incompetent for the purpose. Among the different treatments tried for shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) was found to be most optimal giving an average of 6.7 shoots per explant with mean shoot length of 0.7 cm without any growth of lateral roots within a period of 4 weeks [Figure 1]. MS medium without any plant growth regulator or augmented with BAP alone proved to be ineffective for shoot regeneration. On increasing the concentration of NAA to 2.69 µM and 5.37 µM , an enhanced induction of lateral roots was observed with a marked decrease in shoot bud induction from explants.(Graph 1).

Graph 1: Effect of PGR (µM) Treatment on Adventitious Shoot Regeneration from Root Explants



***In vitro* shoot multiplication**

The media combinations reported to be most optimal in our previous study on *S.chirata* (Pant *et al*., 2010) were MS medium supplemented with different concentrations of BAP, IAA and /or Ads for shoot multiplication and half-strength MS containing IBA for subsequent rooting. Similar combinations were tried in the present study and favourable results were obtained. On transferring *in vitro* grown shoots onto multiplication media, all media combinations tested were capable of inducing multiplication within 2-3 wks. Final observations in terms of mean number of shoots and shoot length were recorded after a period of 4 weeks and 8 weeks. All the treatments were significantly superior to the control except the treatments C4 and C6. In C4 (MS + 13.32 µM BAP) the average shoot number after 4 weeks and in C6 [MS+ 13.32 µM BAP+ 2.85 µM IAA] the average shoot length after 8 weeks was at par with the control. Amongst all, treatment C8 was found to be the best in all parameters. In this combination(MS + 4.44 µM BAP + 2.85 µM IAA + 271.45µM Ads) an average of 10.0 number of shoots with shoot length 2.0 cm after 4 weeks and mean shoot number 18.7 with shoot length 4.0 cm after 8 weeks was recorded [Table1] [Figure 3] .

Table 1: Effect of Plant Growth Regulators on *In vitro* Multiplication of Regenerated Microshoots

Treatment	MS medium +PGR(µM)			Average number of shoots		Average length of shoots(cm)	
	BAP	IAA	Ads	After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
C1	0.0	0.0	0.0	3.5	4	0.7	1.0
C2	4.44	0.0	0.0	4.4	7.4	1.6	2.3
C3	8.88	0.0	0.0	5.4	15	2.3	3.3
C4	13.32	0.0	0.0	5.3	9.1	1.2	1.7
C5	4.44	2.85	0.0	7.3	10.7	1.2	1.8
C6	8.88	2.85	0.0	6.4	9.8	1.1	1.6
C7	13.32	2.85	0.0	6.3	12.4	1.3	2.4
C8	**4.44**	**2.85**	**271.45**	**10.0**	**18.7**	**2.0**	**4.0**
C9	8.88	2.85	271.45	7.4	13.4	1.7	4.6
C10	13.32	2.85	271.45	8.2	11.5	1.9	2.7
Mean				6.4	11.2	1.5	2.5
LSD				1.8	2.8	0.4	1.8

After standardizing the media combination for shoot multiplication, *in vitro* multiplied shoots were subcultured on MS medium fortified with 4.44 µM BAP for maintenance of cultures.

***In vitro* rooting**

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama 1970). For the purpose, regenerated shoots were inoculated onto rooting media. It was observed that root primordia emerged from the shoot base within 10 to 14 days after shoot inoculation in all the treatments. Observations regarding mean root number and root length were recorded after 4 weeks and 8 weeks. All treatments were significantly superior to control. Optimal response was observed in the treatment R4(4.90 µM IBA) with average number of 21.0 roots per shoot after 4-weeks, 28.9 after 10-weeks and average root length 0.9 cm after 4-weeks & 1.6 cm after 10-weeks was observed in [Table 2] [Figure 4].

Table 2: Effect of Different Concentrations of IBA on Rooting of *In-vitro* Regenerated Shoots.

Treatment	1/2 strength MS medium +IBA (µM)	Average number of roots		Average length of roots(cm)	
		After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
R1	0.0	1.6	1.8	0.2	0.2
R2	0.98	6.2	12.1	0.6	1.1
R3	2.46	7.1	13.5	0.8	1.3
R4	**4.90**	**21.0**	**28.9**	**0.9**	**1.6**
R5	9.80	14.0	20.7	0.6	1.1
Mean		10.0	15.4	0.6	1.1
LSD		5.4	6.2	0.3	0.3

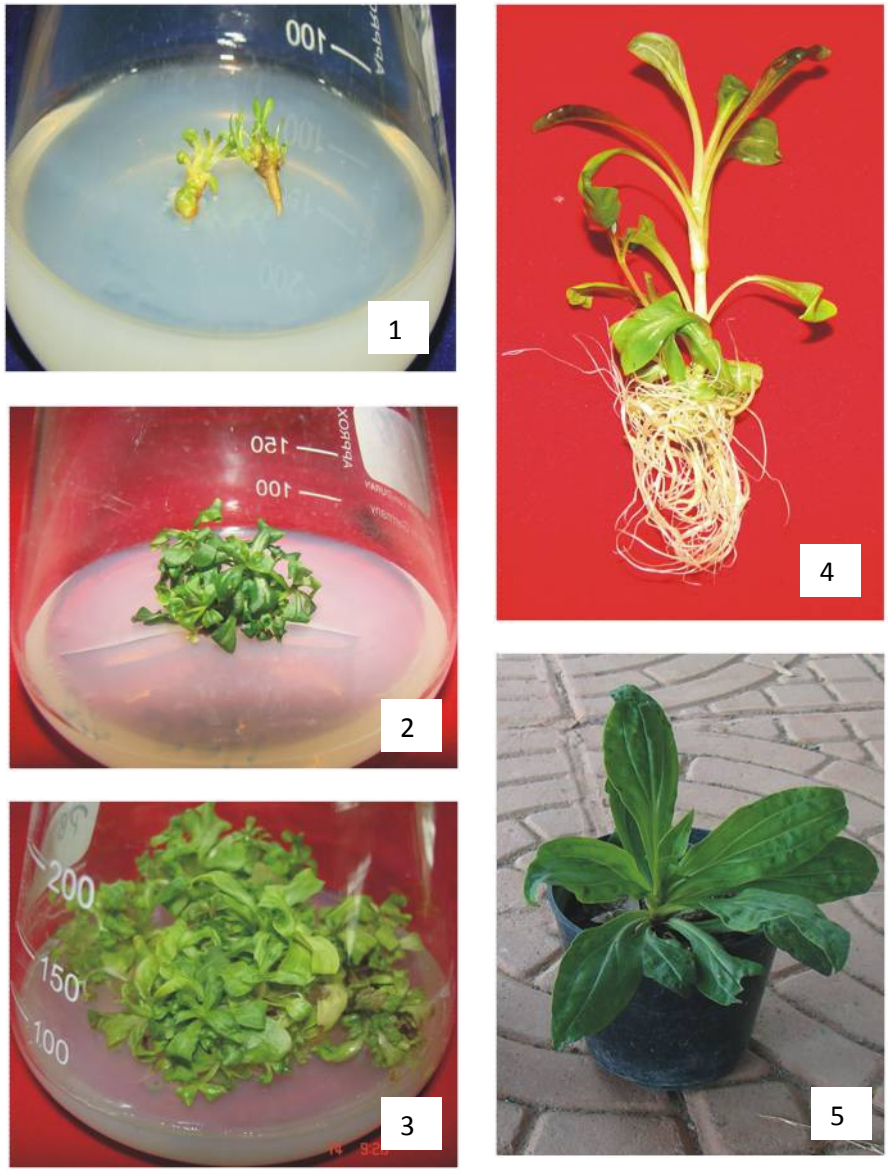


Fig. 1-5 Direct organogenesis of *Swertia chirata* via root explant:
(1) Shoot induction on root segments (2) Culture establishment
(3) Multiplication of shoots (4) *In vitro* rooted plantlet (5) Hardened plantlet.

Hardening and acclimatization

In-vitro hardening was done on liquid 1/4MS medium having 2% sucrose, for 7 days followed by acclimatization in agronet shade house in polybags containing a mixture of soil: sand :manure (1:1:1) and covered with perforated polythene bags. These polythene bags were withdrawn periodically and on emergence of new leaves, the polybags were completely removed. The plants maintained in shade house were irrigated with water. For next one month,

plants were maintained in net house. Well-acclimatized plants were shifted to pots containing soil and gave percentage survival of over 80%. [Figure 5].

Discussion

Careful observations revealed that of the different root segments tried for shoot bud initiation in our study, distal root segments remained non-responsive to shoot bud initiation while proximal and middle segments exhibited shoot regeneration and apparently no difference in the frequency and mode of shoot bud differentiation. Bud initiation and its formation uniformly from the proximal or middle portion of the root indicate that some secondary growth may also be essential for shoot initiation. The results are in corroboration with reports on other species where only the proximal and middle root sections exhibited *de novo* shoot organogenesis in *Comptonia peregrine* (Goforth and Torrey,1977), *Brassica napus* (Sharma and Thorpe, 1987), *Holostemma annulare* (Sudha *et al.*, 2000), *Populus tremula* (Vinocur *et al*.,2000), *Hypericum perforatum* (Zobayed and Saxena, 2003).

In the present study, among the different treatments tried for *in vitro* shoot initation on root segments, a combination of BAP(4.44 µM) and NAA(1.07 µM) gave maximum number of shoots without any growth of lateral roots within a period of 3 weeks . A synergistic effect of BAP and NAA has been highlighted in previous studies on shoot initiation from root explants of *Dalbergia sissoo* (Mukhopadhay and Mohan Ram,1981); *Citrus sinensis* (Burger and Hackett, 1986); *Citrus mitis* (Sim *et al.*, 1989); *Averrhoa carambola* (Kantharajah *et al.*,1992); *Clitoria ternatea* (Shahzad *et al.,*2007) and *Podophyllum hexandrum* (Chakraborty *et al*. ,2010) .

However, in our study, on increasing the concentration of NAA to 2.69 µM and 5.37 µM, significant reduction in shoot bud initiation was observed with an enhanced induction of lateral roots from cut end of roots. Root explants in our study were taken from *in vitro* regenerated roots growing in auxin-rich medium. On placing the explants in another auxin- rich medium may be an inhibiting factor for shoot bud differentiation while facilitating lateral root formation. This is also supported by the view that relatively high concentration of auxin suppresses the inception of shoot buds and promotes growth of lateral roots (Peterson, 1975). Cytokinins are considered to stimulate bud formation and inhibit lateral root formation. There are reports where BAP alone has found to be most efficient for shoot bud regeneration from root tissues in *Citrus mitis* (Sim *et al.,* 1989); *Citrus aurantifolia* (Bhat *et al.*, 1992); *Holostemma annulare* (Sudha *et al*., 2000); *Garnicia indica* (Deodhar *et al.*, 2000); *Blackstonia perfoliata* (Bijelovic *et al*. ,2004); *Crataeva nurvula* (Walia *et al.*,2004); *Melia azedarach* (Vila *et al.*, 2005). Contrastingly, in our study BAP when tried alone at different concentrations was found to be completely inefficient for shoot regeneration.

Wawrosch *et al.,* 1999 reported adventitious shoot regeneration from root explants of *S. chirata* where radicle explants from 3-week-old aseptically grown seedlings were used as primary explants for shoot organogenesis. They optimized a procedure of 3 weeks culturing root explants on BAP(3µM) supplemented MS medium followed by transfer to basal MS medium for another 3 weeks. However, the regeneration procedure described was not very efficient as only 1.9 shoots per explant could be achieved and the regenerated shoots were hyperhydrated. We report a significant improvement over these results as an average of 6.7 shoots per root explants could be obtained in our study which were further multiplied on MS medium supplemented with BAP, IAA and Ads and 18.0 fold multiplication was achieved. The regenerated shoots were healthy and robust without any observation of hyperhydration.

Rooting experiments were initiated after 180 days of subculturing to get maximum material for trying different hormone concentrations. Half-strength MS medium supplemented with IBA at 4.90 µM concentration proved to be most optimal for rooting. The results were similar to our previous findings on *in vitro* propagation of *S. chirata* via nodal explants. Wawrosch *et al.*(1999) had reported callus formation on exposure of shoots to media supplemented with upto 10 µM auxins for rooting. Therefore, they described a method of 2second dipping of shoots in an aqueous solution of 15 ppm NAA followed by 3-4 week cultivation period on hormone-free, half-strength MS medium for root development. In contrast, callusing did not occur in any of the treatments tried for *in vitro* rooting in our study and long, healthy root system developed on IBA augmented medium.

In vitro raised plants are heterotrophic in their mode of nutrition and cannot withstand the environmental conditions without proper hardening and acclimatization, thus, need to be hardened and acclimatized before field transplantation. Regenerated plantlets were hardened under controlled conditions of culture room on ¼ liquid MS medium and sucrose supply was decreased to 2%. After a period of one week, these plantlets were transferred to a rooting mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags in shade. Holes were made in polythene bags for gaseous

exchange. For acclimatizing the plantlets, bags were withdrawn periodically. During the process, the shoots elongated, leaves turned greener and expanded after which the polybags were completely removed. Hardened plants were subsequently transferred to soil filled in pots and over 80 % survivability was recorded.

We suggest that this ability of *S.chirata* plants of direct shoot organogenesis via root in nature, juvenile tissues of the root and specific combinations of growth regulators collectively triggered the process of direct shoot differentiation from root explants *in vitro*.

Conclusion

This study has revealed the morphogenetic potential of *in vitro* grown roots of *S.chirata* as a source tissue for micropropagation. The explants can be easily and regularly obtained from established shoot cultures and do not require disinfection treatment hence being ideal for germplasm exchange and cryopreservation. Normal root culture establishment for a number of plant species have the ability to accumulate secondary products similar to those found in mother donor plant (Tabata *et al.*, 1972; Whitten *et al.,* 1981). In *S.chirata* root being the most important part of the plant for medicinal purposes holds importance as the starting material for tissue culture studies. The present protocol will therefore serve as alternative means for *in vitro* clonal propagation of this endangered plant with immense medicinal value and further biochemical and physiological investigations.

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