**INTRODUCTION**

*Swertia chirata* (family: Gentianaceae) commonly known as chirata is an important medicinal plant with immense potential. A native to temperate Himalaya (altitude above 4,000-10,000 ft.), the species is found from Kashmir to Nepal, Bhutan and Khasi hills.

*S. chirata* is utilized for its bitter bioactive compounds which include gentianine alkaloids, amarogentin, xanthones and glycosides (Dalal and Shah, 1956; Charavarty et al., 1991; Chakravarty et al., 1992; Jensen and Schripsema, 2002). The bitterness, antihelminthic and anti-diarrhoeal properties are attributed to amarogentin and other active principles of the herb (Dalal and Shah, 1956).

The entire plant is used in traditional medicine but the root is said to be the most powerful part which is used for treating several health problems (Watt, 1972; Bajaracharya, 1979; Kirtikar and Basu, 1984).

*S. chirata* is much prized as a bitter tonic without aroma and astringency. In Indian medicine, it is prescribed in a variety of forms and combinations in chronic fever, anemia and is used as special remedy for bronchial asthma and liver disorders. It is credited with febrifugal, laxative, stomachic, insecticidal, antihelminthic and anti-diarrhoeal properties. Extracts of *S. chirata* are also used as hepatoprotective agents and anti-inflammatory, antifungal, antimalarial and anticarcinogenic properties of this medicinal plant are also known (Kirthikar and Basu, 1984; Keil et al., 2000; Brahmachari et al., 2004; Ravishankar et al., 2007; Mallikarjun et al., 2010). The bitter infusion of the plant is used as a blood purifier, for skin diseases and as bitter tonic for fever and indigestion. The presence of xanthones in the species is reported to be used for treatment of tuberculosis and also acts on the central nervous system as a stimulator (Ghosal et al., 1973).

Herbal medicines such as Ayush-64, Diabecon (Himalayan herbal care) and Melicon v ointment (Cadilla pharmaceuticals) contain *chiretta* extracts in different proportions for its multiple medicinal properties (Mitra et al., 1996; Valecha et al., 2000).

There is an increasing pharmaceutical demand of the species both, in the indigenous and the world market. Extensive collection and unscientific harvesting practices of the plants from the natural habitat leads to an increasing danger of extinction (Bhattarai and Shrestha, 1996; Joshi and Joshi, 2008). According to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria, *S. chirata* has been categorized as critically endangered (Joshi and Dhawan, 2005). This leads to an urgent need for conservation of the plant. *S. chirata* has also been
The present investigation was undertaken with the objective of developing a simple and efficient methodology for rapid and mass multiplication of *Swertia chirata* using root segments of *in vitro* raised seedlings and claimed that adventitious shoot regeneration from root segments was more effective than through shoot multiplication. Ahuja et al. (2003) patented a protocol for rapid shoot multiplication from field-grown nodal explants of *S. chirata*. Joshi and Dhawan (2007a, 2007b) reported shoot multiplication from *in vitro* grown seedlings and regeneration via immature seed cultures. Balaraju et al. (2009) published reports on *in vitro* propagation of *S. chirata* from nodal meristems of field-grown plants, direct shoot multiplication using *in vitro* leaves and regeneration via immature seed cultures. Chaudhuri et al. (2007; 2008; 2009) published reports on *in vitro* propagation of *S. chirata* from nodal meristems of field-grown plants, direct shoot multiplication using *in vitro* leaves and regeneration via immature seed cultures. Koul et al. (2009) gave a protocol for complete plant regeneration via nodal segments and axenic leaf culture. Their study was supplemented with molecular characterization and marker glycoside assessment of *in vitro* raised plants of *S. chirata*.

The present investigation was undertaken with the objective of developing a simple and efficient *in vitro* methodology for rapid and mass multiplication of *Swertia chirata* through axillary shoot sprouting.

**MATERIALS AND METHODS**

**Source of explants**

Donor plants of *Swertia chirata* were collected from their natural habitat of Chakrata, Uttarakhand (altitude 7,699 ft., lat. 30°43.642’, long. 77°51.941’) during the month of May, 2007. Nodal explants were taken as the starting material for *in vitro* culture initiation.

**Explant treatment**

Stem segments with nodes were cut and washed in running tap water followed by soaking in liquid detergent for 5 min (Teepol, Reckitt and Colman, India 5-6 drops/100 ml) in a vial under gentle agitating conditions. Subsequently these were washed with distilled water to remove traces of detergent. This was followed by a treatment with surfactant Tween-20 (4-5 drops/100ml) and re-washing with distilled water. After washing, nodal segments were excised from disinfected material and cut to 2-3 cm length with at least one node in each segment. The nodal explants were surface sterilized with 0.1% (w/v) HgCl₂ (Himedia) for 7 min. under laminar-air flow cabinet and thoroughly rinsed 4-5 times with autoclaved distilled water.

**Culture conditions and *in vitro* establishment of plantlets**

For establishment of cultures, surface disinfected nodal explants were inoculated on to full strength MS (Murashige and Skoog, 1962) basal medium having 3% sucrose, solidified with 0.7% (w/v) agar (Himedia) and supplemented with different concentrations and combinations of PGRs viz. BAP (2.22 µM - 22.20 µM), Kn (2.32 µM - 23.23 µM), GA₃ (0.29 µM - 1.44 µM) and IAA (1.14 µM - 11.40 µM). The pH of the medium was adjusted to 5.8 using 1 N NaOH or 1N HCl prior to adding agar and dispensed in 10 ml aliquots into 150 x 20 mm culture tubes (Borosil, India). The culture medium was autoclaved at 120 °C and 1.0 x 10⁷ Pa for 15 min. Cultures in all experiments were incubated in culture room at 23 ± 1 °C and 60-65% relative humidity under a 16 / 8 hr (light / dark) photoperiod with light supplied by cool-white fluorescent tubes (Philips, India) at an intensity of 50 µmol m⁻² s⁻¹.

**Induction of multiple shoot buds from nodal meristems**

Single shoots were separated from established cultures and inoculated onto multiplication medium in conical flasks (250 ml, Borosil, India). Full strength MS basal medium supplemented with varying concentrations of cytokinins BAP (4.44 µM - 13.32 µM) or Kn (4.65 µM-13.75 µM) alone and in combination with auxin IBA (0.98 µM - 2.85 µM) and / or adjuvant Adenine Sulphate (271.45 µM) was tested for multiplication of *in-vitro* proliferated shoots. MS medium devoid of any PGR was used as control.

The number of shoots developed and shoot length was recorded after a period of 4 wks and 8 wks. Sub culturing was carried out at periodic intervals of 3 wks.

**Rooting of microshoots**

*In-vitro* developed shoots having 1 or 2 nodes were excised and transferred to root induction medium comprising of 1/2 strength MS medium with 2% sucrose and supplemented with different concentrations of IBA (0.98 µM - 9.80 µM), IAA (1.14 µM - 11.40 µM) and NAA (1.07 µM - 10.74 µM). Data on the number of roots per shoot and root length was scored after a period of 4 wks and 8 wks.

**Hardening and acclimatization**

For *in vitro* hardening, rooted shoots were transferred to 1/4 MS strength medium having 2% sucrose devoid
In vitro propagation through axillary bud culture of *S. chirata*

of PGR for 7 days in flasks. Thereafter, they were transferred to polybags containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags and kept under agronet-shade house conditions. Acclimatized plants were later shifted to soil in pots.

**Statistical Analyses**

Experiments were done in triplicate and each treatment consisted of minimum ten replicates. The data was analysed using 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 Significant Difference (LSD).

**RESULTS**

**Initiation and multiplication of shoot cultures**

Bud break in nodal explants was achieved on MS medium supplemented with BAP (13.32 µM) + IAA (2.85 µM) + GA₃ (1.16 µM) within 7-10 days of inoculation (Fig. 1). The regenerated shoots were transferred onto fresh medium of same composition to establish an initial stock of shoots (Fig. 2). These *in vitro* grown shoots were subsequently used in the second set of experiments to assess the effect of various PGRs on shoot multiplication summarized in Table 1 [Supplementary data]. In control, the shoots did not exhibit any growth and eventually necrosed. All the other media combinations tested were capable of inducing multiplication within 2-3 wks. Regarding average number of shoots per explant it was studied that treatments T3, T16 and T19 (for observations after 4 weeks) and treatments T3, T17 and T19 (for observations after 8 weeks) proved to be at par with the control. All the other treatments were significantly superior to control. Similarly, results obtained for average shoot length (cm) after 4 wks and 8 wks showed that except T3 (at par with control) all the treatments were significantly superior to control.

For all the parameters studied, it was observed that treatment T13 (MS + BAP 4.44 µM + IAA 2.85 µM + Ads 271.45µM) proved to be the best and varied significantly from rest of the treatments. In this combination a maximum of 11.8 and 18.5 mean number of shoots per explant was observed after a period of 4 wks and 8 wks, respectively. The same combination also gave the best results for average shoot length recorded as 1.9 cm after 4 wks and 2.6 cm after 8 wks (Fig. 3). MS medium supplemented with 4.44 µM BAP was used for routine sub culturing of multiple shoots at an interval of every 3 wks. By this procedure, 50 sub cultures have already been carried out without observing any hyperhydration. The rate of shoot formation per culture increased 10-15 folds after 4th to 5th subculture. The multiplication rate did not decline over a period of three years of maintenance of cultures.

**In vitro rooting**

In our study, a consistent rooting was noted in all the three auxins viz. IBA, IAA and NAA within 7-10 days of culture without any intervening callus phase. Root primordia emerged from the shoot base within 10-15 days after shoot inoculation and soon after that root growth was rapid. Overall, treatment R11 (4.90 µM IBA) was most optimal in all rooting parameters and
In the present study, complete plantlets of *S. chirata* were obtained through *in vitro* culture of nodal explants collected from field-grown material. Bud break was achieved only in one treatment comprising of full strength MS medium + BAP + IAA + GA₃. No other hormonal combination induced axillary shoot sprouting and eventually necrosis was observed. GA₃ has previously been reported to stimulate shoot induction from nodal explants in *Gentiana triflora* (Zhang and Leung, 2002). The same media combination was used for establishment of an initial stock of shoots to carry out further experiments on multiple shoot proliferation.

Among the combinations of cytokinins tested for shoot multiplication, BAP was found to be more efficient. Superiority of BAP over Kn in *in vitro* shoot multiplication has been reported in *Gentiana kurroo* (Sharma et al., 1993), *Chlorophyllum bortvillianum* (Purohit et al., 1994), *Rotula aquatic* (Martin, 2003), *Orthosiphon stamineus* (WaiLeng and LaiKeng, 2004). In the present study, addition of exogenous auxin IAA to cytokinin supplemented medium promoted axillary shoot proliferation from nodal explants and also enhanced shoot multiplication. The positive effect of auxin at lower concentration might be due to the nullification of higher concentration of cytokinins and promotion of shoot elongation (Hu and Wang, 1983). The synergistic effect of BAP and IAA in efficient shoot multiplication has been reported in several other medicinal plants including *Gentiana sps* (Momcilovic et al., 1997), *Leptadenia reticulate* (Arya et al., 2003), *Centaurium erythraea* (Piatczaka et al., 2005), *Ruta graveolens* (Bohidar et al., 2008).

In a previous report on *in vitro* shoot regeneration from nodal explants of *S. chirata* (Chaudhuri et al., 2007) an optimal response for multiple shoot induction and further multiplication was achieved on 1/2 strength MS medium augmented with 4.65 µM Kn + 0.44 µM BAP + 75 mg/l CH + 10mm KNO₃. On this medium an average of 18.3 shoots per explant were obtained after 10 wks period. Joshi et al. (2007) reported a maximum shoot multiplication of 4.5 fold after 4 wks from seedling derived nodal explants on MS medium + 4 µM BAP + 1.5 µM 2iP. The efficiency of combination of BAP and Kn in multiple shoot induction and multiplication from shoot tip explant (Balaraju et al., 2009) and nodal explants (Koul et al., 2009) has also been reported in *S. chirata*. In our study, a stimulating effect of a combination of BAP, IAA and Ads on shoot multiplication was observed. Adenine sulphate has been reported to show a synergistic effect with other cytokinins; stimulating cell growth and gently enhancing shoot formation (Raha and Roy, 2001). The efficiency of adenine sulphate in promoting shoot multiplication has previously been highlighted in the species such as *Psoralea coryfolia* (Saxena et al., 1997), *Pentamena indicum* (Sivanesan et al., 2007), *Jatropha curcas* (Datta et al., 2007).

It has previously been shown that decrease in sucrose concentration in the medium enhanced photosynthetic ability of plantlets (Desjardins et al., 1995). Consequently, sucrose concentration was decreased to 2% to make the plants more autotrophic. Since the concentration of nitrogen ions required for root formation is much lower than for shoot formation (Driver and Suttle, 1987), in the present study half-strength media was used for rooting. Role of auxins in development of roots is well established and reviewed (Scott, 1972). In the present study also, shoots cultured in auxin-free medium (control) failed to form roots. IBA at 4.90 µM concentration proved to be most optimal for rooting.

Our results also present an improvement over previous reports by Chaudhuri et al. (2007; 2008) who reported RIM (root induction medium) consisting of 1/2 MS + 5.71 µM IAA + 4.90 µM IBA to give best result of an average number of roots of 12.9 and root length 6.7 cm

**DISCUSSION**

The synergistic effect of BAP and IAA in efficient shoot multiplication has previously been highlighted in the species such as *Psoralea coryfolia* (Saxena et al., 1997), *Pentamena indicum* (Sivanesan et al., 2007), *Jatropha curcas* (Datta et al., 2007).

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**Hardening and acclimatization**

*In vitro* raised plants are heterotrophic in their mode of nutrition need to be hardened and acclimatized before field transplantation. Plantlets developed via axillary bud culture were hardened *in-vitro* by placing them on liquid 1/4 MS medium without PGR and having 2% sucrose for 7 days. Plantlets with well-developed shoots and roots (Fig. 5) were subsequently transferred to a rooting mixture of soil: sand: manure (1:1:1). These were covered with perforated polythene bags in shade and maintained in Net house for next 30 days where they exhibited enhanced growth. When transferred to soil in pots, survival rate was over 85% (Fig. 6).

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in a period of 4 wks. For shoots regenerated from callus via immature seeds, optimal rooting media was standardized as 1/2 MS + 9.8 µM with 3% sucrose giving an average of 4.2 roots per shoot after 4 weeks (Chaudhuri et al., 2009). Joshi et al. (2007) optimized a rooting medium comprising of 1/2 MS + 1 µM NAA + 500 mg/l Activated charcoal giving an average of 6.5 roots with root length 8.69 cm in 4 wks. In their study, the shoots inoculated on medium without activated charcoal developed calli at the base of shoot and roots remained thick, small and tuberous. No such observations were recorded in our study and a higher number (35.3 after 4 wks, 37.5 after 8 wks) of long and healthy roots developed.

Ahuja et al. (2003) described efficiency of MS medium fortified with IAA ranging between 1-5 mg/l (5.71-28.57 µM) for 60-65% rooting in a duration of 8 wks whereas in our study 75-90% rooting was obtained with IBA (4.90 µM) a maximum of 92% rooting could be achieved. On medium supplemented with IAA ranging between 1-5 mg/l (5.71-28.57 µM) for 60-65% rooting in a duration of 8 wks. In their study, whitish, short and well developed roots with root hair sparsely developed roots. However, in our study thick, small and tuberous. No such observations were recorded in our study and a higher number (35.3 after 4 wks, 37.5 after 8 wks) of long and healthy roots developed.

Induction of multiple shoots through axillary branching is now recognized as a useful technique for propagation and in-vitro conservation of threatened plants, especially those in which roots or rhizomes contain the active compound (Constabel, 1990; Sharma and Chandel, 1992). We have presented here a simple and reproducible procedure for production of a critically endangered species - *S. chirata*. The method described has been used for recurrent shoot production without loss of multiplication potential in more than three years of 3-week sub culture. Thus, this package of technology has the potential for increased productivity on mass scale. The protocol can be used for raising genetically uniform population for sustainable supply of plant materials to pharmaceutical industries and conservation of elite germplasm.

**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:** Gibberellic acid; **Ads:** Adenine sulphate; **PGR:** Plant growth regulator

**References**


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**In vitro** propagation through axillary bud culture of *S. chirata*
In vitro propagation through axillary bud culture of Swertia chirata


