



Regeneration and characterization of *Swertia chirata* Buch.-Ham. ex Wall. plants from immature seed cultures

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ABSTRACT

First generation immature seeds (R_1) were collected from a field transferred micropropagated plant and seeds were induced to develop organogenic calli in *Swertia chirata*, a traditional revenue earning medicinal plant. Half strength MS medium with different growth regulators namely, BA, Kn (2.22–4.44 μ M), NAA (2.69–5.37 μ M), and 2.26 μ M 2,4-D were used to induce callus and organogenesis. Isolated shoots produced roots either in the same medium or in presence of NAA (2.69–10.74 μ M) or IBA (2.46–9.8 μ M). Fully developed plantlets were successfully transplanted to soil and the fertile seed bearing plants developed. Occasionally plants derived from more than 56 weeks old calli showed some morphological variations. Such variations in regenerated plants is not reflected in their chromosomal constitution, with normal $2n = 26$ chromosomes. Likewise, no variation was observed in DNA fingerprinting patterns among the short-term raised culture regenerants, which were morphologically similar to that of the donor plant illustrating their genetical uniformity and clonal fidelity. On the contrary, variation in DNA fingerprinting patterns was observed in long-term culture raised plants.

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1. Introduction

The growing worldwide demand for traditional medicinal plants has made large-scale commercial cultivation and genetic improvement imperative. *Swertia chirata*, commonly known as chirata is one such plant, which has great therapeutic potentials. It is generally used as blood purifier in liver diseases and is also valued for its anti-inflammatory, antiplatelet, anticancerous, antifungal and antimalarial activity (Brahmachari et al., 2004). *Swertia* also acts on the central nervous system as a stimulator (Ghosal et al., 1978). *S. chirata* grows wild in different hilly terrain of India, Bhutan and Nepal, while other species of *Swertia* namely, *S. japonica* and *S. pseudochinensis* are reported from Japan and China, respectively. Plants of *S. chirata* are becoming critically endangered due to extensive habitat loss from the wild, poor seed germination rate, low viability, long gestation period and lack of adequate commercial plantation (Anonymous, 1997; Rai et al., 2000; Joshi and Dhawan, 2005).

Keeping these in view, we have undertaken an investigation on formulating alternate strategies for the conservation of *S. chirata* through *in vitro* propagation.

Miura et al. (1978a,b) had initiated callus mediated shoot differentiation from young roots of sterile seedlings of *S. japonica*, but failed to induce roots in the regenerated shoots. However, he succeeded in raising complete *S. pseudochinensis* plant regeneration under *in vitro* condition using seedling-hypocotyls and roots as explants. Wawrosch et al. (1999) have used root segments from the germinated seeds of Nepalese *S. chirata* for shoot differentiation. A patent on micropropagation protocol of *S. chirata* has been granted to Ahuja and his co-researchers (2003). While Joshi and Dhawan (2007a,b) have reported *in vitro* multiplication of *S. chirayita* from nodal explants of the seedlings and checked their clonal fidelity through ISSR markers.

It appears that all the previous researchers have employed seeds from natural populations in their studies. On the other hand, in the course of our systematic effort to explore alternative strategies for conservation of *S. chirata*, we have reported (Chaudhuri et al., 2007) production of genetically true to type fertile plants from nodal meristems of *S. chirata* growing in the natural habitat almost simultaneously with Joshi and Dhawan (2007a). We have achieved direct organogenesis from leaf segments of *in vitro* grown plantlets and their successful transfer

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Abbreviations: BA, 6-benzyladenine; IBA, indole-3-butyric acid; Kn, kinetin; MS, Murashige and Skoog medium; NAA, α -naphthalene acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; PGR, plant growth regulator.

to the field conditions (Chaudhuri et al., 2008). In both the cases clonal fidelity of the regenerants were checked using DNA fingerprinting patterns.

Annual plants of *S. chirata* produce large number of minute seeds (~300 μm in diameter) packed within a capsule type of fruit with poor seed germination rate (2–4%). In this communication, we report the use of immature seeds from a genetically stable micropropagated plant for *in vitro* regeneration of *S. chirata* plants and succeeded in obtaining callus mediated plant regeneration. As calli represent large number of unorganized mass of heterogeneous cells it often lead to the production of somaclonal variants (Larkin and Scowcroft, 1981). Characterization of regenerated plants at the molecular level is of utmost importance because chromosomal analysis alone is insufficient to detect variations at the structural level especially in *S. chirata*, where chromosome number is $2n = 26$ and sizes are too small ranging from 2 to 4.4 μm .

In view of the fact stated above the objectives of this investigation was to (i) formulate a reproducible protocol for *in vitro* propagation exploiting potentialities of immature seeds and (ii) chromosomal and DNA fingerprinting analyses of the culture regenerants to check the clonal fidelity in both short- and long-term callus raised plantlets.

2. Materials and methods

2.1. Plant material

Immature capsules (0.5–0.8 cm) were collected during the month of November, 2003 from a tissue culture regenerated plant of *S. chirata* (Buch.-Ham. ex Wall.). These were washed thoroughly and treated with 5% (v/v) Teepol (commercial bleach) for 30 min, followed by surface sterilization with 0.1% HgCl_2 for 25–30 min. Sterilants were removed from the capsule surface by repeated washing with sterilized distilled water. Intact capsules and excised immature seeds were placed on nutrient medium. Each experiment was repeated thrice with 5 replicates.

2.2. Culture media and culture conditions

Full or half strength Murashige and Skoog's (MS, 1962) medium with 3% (w/v) sucrose, 0.7% bacto-agar and different combinations and concentrations of plant growth regulators (PGRs) namely, BA, Kn, 2,4-D and NAA were used to induce calli from the immature seeds. The pH of culture media was adjusted to 5.6 before autoclaving for 15 min at 101 °C at 1.05 kg cm^{-2} . All cultures were incubated at 22 ± 2 °C, under 16 h photoperiod with a light (photosynthetically active radiation) intensity of 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (400–700 nm) provided by 40 W cool white fluorescent tubes followed by 8 h dark period. All experiments were repeated three times.

2.2.1. Initiation of calli

Immature capsules and isolated R_1 seeds were placed in full and 1/2 MS medium with 3% (w/v) sucrose with different combination of PGRs, such as, BA, Kn (2.22–4.44 μM), 2,4-D (2.26 μM) and NAA (2.69–5.37 μM) for induction of calli. Frequency of callus induction was scored after 6 weeks of incubation in both the above-mentioned salt concentrations of MS medium containing different combinations and concentrations of PGRs as stated and data were scored.

2.2.2. Induction of shoot

Calli were maintained in half strength MS medium supplemented with 2.26 μM 2,4-D and 2.22 μM Kn. Usually, calli were subcultured at an interval of 8 weeks in the same medium. In

addition, during each subculture, one set of calli was transferred to shoot induction medium containing equimolar BA and Kn either 2.22 or 4.44 μM each with (0.54–1.6 μM) NAA, while another set was transferred to half strength MS medium supplemented with equimolar amount of BA and Kn, either 2.22 or 4.44 μM . Regeneration efficiency of 8–80-week-old calli (i.e. up to 9th subculture) was studied. Regenerants obtained from calli upto 56 weeks old or more are referred here as short-term regenerants (STRs) and long-term regenerants (LTRs), respectively. All the data on shoot development were taken after 6 weeks of subculturing.

2.2.3. Induction of root and maintenance of complete plant

About 4–5 cm long shoots were placed in root induction media (RIM). The medium was 1/2 MS salts with 3% (w/v) sucrose and supplemented either with NAA (2.69, 5.37 and 10.74 μM) or with IBA (2.46, 4.9 and 9.8 μM). The shoots were kept for 4 weeks in the RIM and rooted shoots were maintained in 1/2 MS basal medium with 0.5% (w/v) sucrose. Frequency of root induction in auxin containing medium and number of roots developed per shoot was recorded.

2.2.4. Transplantation and hardening of regenerants

Plantlets with well-developed roots were thoroughly washed in tap water and transplanted in earthen pots containing soil (clay:sand:compost; 1:1:1 at pH 5.8) and were covered by polythene bags and allowed to grow for 2 weeks under 80–90% humidity. Subsequently, bags were removed for 2–3 h every day for gradual acclimatization and finally covers were completely removed from the plantlets after 1 week. Hardened plants were transplanted and grown at Darjeeling during the calendar year 2005 and 2006.

2.3. Study of leaf-morphology among the culture regenerants

Variations in the leaf characters were apparent among the culture regenerants. Number of leaves per plant, length and breadth of leaves, phyllotaxy, stomatal frequency and size of stomata were studied from ~3 months old STRs and LTRs. All the cultures were maintained in half strength MS basal medium with 0.5% sucrose.

2.4. Analysis of experimental data

Statistical difference between mean tabulated values were estimated ($P \leq 0.05$) using Duncan's multiple range test (DMRT) and Tukey's HSD test with the Statistica Software v 5.0 (StatSoft, 1995).

2.5. Chromosome analysis

Shoot tips were excised from more than 100 randomly selected STRs and LTRs, grown under *in vitro* condition, in order to check the chromosome numbers from the metaphase stage of dividing cells. Young shoot tips were treated with saturated solution of para dichloro benzene (PDB) for 4 1/2 h at 14 °C and were kept in Carnoy's solution (acetic acid:chloroform:ethanol; 1:3:6) for overnight. Hydrolysis of the shoot tips was performed with 1N HCl at 60 °C for 8–10 min. Hydrolysed tissues were stained with 2% aceto-orcin for 4 h and squashed in 45% acetic acid on the glass slides to obtain well-scattered metaphase plates. The slides were observed under the high power of light microscope and chromosome numbers per cell were manually counted.

2.6. PCR compatible DNA isolation and generation of DNA fingerprinting pattern

Genomic DNA were isolated from young leaves of donor *S. chirata* plants and from both STRs and LTRs. DNA fingerprinting

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