



Plant regeneration through direct and indirect organogenesis, phyto-molecular profiles, antioxidant properties and swertiamarin production in elicited cell suspension cultures of *Swertia minor* (Griseb.) Knobl

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Abstract

The present study reports an optimized protocol for high frequency in vitro plant propagation through direct and indirect organogenesis, phytochemical accumulation, molecular profiling and antioxidant evaluation for micropropagated *Swertia minor*, a promising alternative to industrially important *Swertia chirayita*. Moreover, the study also aimed at enhancing the production of antidiabetic and anti-obesity drug swertiamarin using an alternative technology of elicited cell suspension cultures. Different types, concentrations and combination of cytokinins and auxins showed their effects during various in vitro growth stages. A combination of BAP (3.0 mg/l) and TDZ (1.0 mg/l) had a dominant role in promoting multiple shoot proliferation with production of an average of 19.1 ± 0.95 shoots/node in 85% response. MS medium added with IBA (2.0 mg/l) showed optimal response for in vitro rooting (9.2 ± 0.56 roots/shoot). In order to establish genetic stability, molecular marker-based profiling of micropropagated plants were done and 'monomorphic banding pattern were identical to the mother plants. 2,4-D (2.0 mg/l) supported the maximum callus induction and proliferation rate (95%). The wild-grown plants showed higher polyphenols content and antioxidant activities as compared to callus and in vitro derived plantlets. However, chitosan-treated (25 ppm) methanolic extract of cell biomass accumulated in cell suspension cultures produced higher contents of swertiamarin (1.45 mg/g DW) than salicylic acid and methyl jasmonate. The described protocol can be effectively used for the large-scale propagation, exploitation of active compounds and will serve as potential alternative to *S. chirayita* for fulfillment of over-growing industrial requirements.

Key message

The present investigation addressed in vitro regeneration, callus culture, somatic embryogenesis, molecular profiling, secondary metabolite production, cell suspension culture studies for first time in *Swertia minor*.

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Keywords Callus · Cell suspension · Elicitation · Genetic fidelity · Plant regeneration · Swertiamarin

Abbreviations

ABTS	2, 2-azino-bis (3-ethylbenzo-thiazoline-6-sulphonic acid)
AEAC	Ascorbic acid equivalent antioxidant capacity
DMPD	N, N-dimethyl-p-phenylenediamine
DPPH	1, 1- diphenyl-1-picryl hydrazyl
DW	Dry weight
FRAP	Ferric reducing antioxidant power
FW	Fresh weight
g	Gram
MeJA	Methyl jasmonate
mg	Milligram
MS	Murashige and Skoog's medium
PGR(s)	Plant growth regulator(s)
RF	Regeneration frequency
RP-UFLC	Reverse phase-ultra flow liquid chromatography
RSA	Radical scavenging activity
SA	Salicylic acid

Introduction

Industrial demand for medicinal plants is increasing owing to worldwide awareness in herbal sector engaged in production of herbal health care formulations. The genus *Swertia* L. is among the top ranked medicinally important plant group belonging to family Gentianaceae. The genus comprises over 170 species which are primarily distributed in temperate regions of the northern hemisphere (Brahmachari et al. 2004). About 40 species of *Swertia* are found in India, mainly at high altitude (1200–3000 m) in the temperate Himalayan region ranging from Kashmir to Bhutan, and also in the Khasi and Western Ghats hills (Anonymous 1982; Scartezzini and Speroni 2000).

Swertia chirayita is one of the potent medicinal plants of this genus, known for its numerous health beneficial and pharmacological implications (Kumar and Van Staden 2016; Kshirsagar et al. 2019). Due to this medicinal importance and good domestic and international market, over-exploitation of chirayita occurs, hence, existing population of chirayita is diminishing and now chirayita has been categorized as critically endangered. Due to its high demand and scarcity, trade of chirayita is affected by adulterants and to overcome this problem, in recent study from our research group investigated the DNA barcoding approach to identify the adulterants (Kshirsagar et al. 2017a). Recently, in search of potential alternative to chirayita, Kshirsagar et al. (2015a, 2016, 2017b, 2020) evaluated the phytochemical potential, seco-irridoid glycosides and xanthonoid content for Indian *Swertia* species. As a result, *Swertia minor* (Griseb.) Knobl

was found with maximum content of swertiamarin and hence it could be used as potential alternative to *S. chirayita*. Moreover, *S. minor* possesses wide range of biological activities including antioxidant, anti-hyperglycemic and anti-glycation properties (Chokkalingam et al. 2012; Kshirsagar et al. 2014). However, biotechnological implication especially plant cell, tissue and organ culture could be a possible strategy for exploitation of full potential of *S. minor*.

The objective of the current study was to develop plant regeneration protocol *via* shoot organogenesis (direct and indirect), somatic embryogenesis and genetic diversity assessment of regenerants. In addition, the phytochemical and pharmacological properties of *in vitro* grown plant were compared to callus and the pre-dominantly used wild plants using different solvent systems and extraction methods. Moreover, cell suspension cultures were optimized using various elicitor treatments for biosynthesis of swertiamarin and quantified using reverse phase-ultra flow liquid chromatography (RP-UFLC) analysis.

Materials and methods

Plant material, culture conditions and shoot production

The mature fruits of healthy plant containing seeds were brought from Panhala locality of the Northern Western Ghats. The seeds were washed with tap water for two times were treated with 0.1% mercuric chloride for 2 min and then washed twice with distilled water. MS basal liquid medium (Murashige and Skoog 1962) was used for wetting the cotton during seed germination. The culture tubes having medium (pH adjusted to 5.8 ± 0.2) or wet cotton were autoclaved at 121 °C for 20 min. All the cultures were maintained at 25 ± 1 °C with 16-h photoperiod provided by cool fluorescent lamps ($35 \mu\text{mol}/\text{m}^2/\text{s}^1$). Shoot tip excised from *in vitro* grown seedlings were inoculated in the MS medium supplemented with various concentrations of BAP, TDZ either alone or their combinations. Sub-cultures were done after 4 weeks of interval.

Callus induction, indirect shoot organogenesis and embryogenesis

Leaves of *in vitro* grown plantlets were used as source of explants for callus induction. The explants were inoculated on the MS medium supplemented with singular supplementation of either 2,4-D (1.0–5.0 mg/l) or NAA (1.0–5.0 mg/l). In order to induce shoots from calli, various treatments of BAP (1.0–5.0 mg/l) were tested in combination with 2,4-D

(2.0 mg/l). Different concentrations of 2,4-D (1.0–2.0 mg/l) in combination with varied treatments of NAA (0.5–2 mg/l) were tested for induction of somatic embryos.

In vitro rooting, acclimatization and ex vitro establishment of regenerants

Microshoots with well-developed shoot system (4–5 cm) were transferred to MS medium supplemented with various concentrations of IBA, IAA and NAA for in vitro root induction. Data of the explant response mean number of shoots, roots and its length were recorded after 4 week of incubation period. The plantlets with fully grown shoots and roots were taken out from the culture medium, washed to remove the traces of medium were transferred to plastic pots (12–3/4 in. D × 14–1/2 in. H) containing a mixture of sterile garden soil, river sand and organic manure (1:1:1). The pots were covered with polythene bags and supplemented with ¼ MS basal salt solution on alternate day for first week. The potted plantlets were maintained in culture room at 25 ± 2 °C for 2 weeks before transferred to glass house [progressive increase in temp as 27, 30 and 35 °C with 40 µmol/m²/s¹ light irradiance (16 h)]. After 4 weeks' interval in glasshouse, the plantlets were transferred to the soil for self-perpetuation.

Genetic fidelity analysis

Optimization of PCR conditions for random amplified polymorphic DNA (RAPD) assay

A total of 25 random decamer primers (Bangalore Genei Ltd. India) were screened for RAPD analysis. The protocol for RAPD analysis was adapted from that of Kshirsagar et al. (2015b) and Chavan et al. (2013) with little modification. The reaction mixture consists of 10× PCR buffer (2.5 µl), 2.5 mM MgCl₂ (1.5 µl), 100 mM dNTPs (2.0 µl), primer (2.0 µl), Taq polymerase (0.2 µl), 40 ng of template DNA (2.0 µl), and 14.8 µl sterile distilled water. PCR was performed at initial temperature of 94 °C (6 min, 1 cycle), followed by 38 cycles of 30 s at 94 °C, 30 s at 36 °C and 2 min at 72 °C, and a final cycle of 10 min at 72 °C. Initially, a total of 25 RAPD primers were used to amplify the genomic DNA, however 18 were selected for further studies depending upon their amplification, scorability and reproducibility of bands.

Agarose gel electrophoresis, data scoring and analysis

After PCR amplification, the products were resolved on agarose gel (1.5%) prepared in 1 × TAE buffer and stained with ethidium bromide. The electrophoresis was carried out in horizontal gel electrophoresis apparatus at 50V for 2–3 h i.e. till the bromophenol blue dye travelled at least 2/3rd length

of gel. 100 bp ladder was used as a DNA marker (GeneRuler, Thermo Scientific, India). The gel was photographed by using gel documentation system (BioRad, Hercules, CA). The bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. Prominent and reproducible bands obtained for each RAPD primer were considered.

Phytochemical and antioxidant analysis

Plant material and extract preparation

Naturally grown plants, in vitro grown callus and in vitro raised plantlets were cut into pieces, dried at room temperature under shade for 10 days and grinded to fine powder using a Wiley mill. The extracts were obtained in various solvents (methanol, ethanol and water) using various extraction techniques *viz.* static extraction (SE) for 24 h; continuous shaking extraction (CSE) for 24 h and ultra-sonication extraction (UE) for 15 min.

Determination of total phenolics and flavonoids content

The materials and methods were used as previously reported by our work (Kshirsagar et al. 2015a) for experiments like evaluation of total phenolic content (TPC), total flavonoid content (TFC). The content of total phenolics was expressed as milligrams of gallic acid (GAE) and tannic acid (TAE) equivalent per gram dry weight (DW), while the values of total flavonoids was expressed as milligrams of rutin (RE) and quercetin (QE) equivalent per gram dry weight.

Determination of antioxidant potential using different assays

Antioxidant potential of callus, in vitro- grown and wild grown plants were assessed using four different assay such as 1, 1- diphenyl-1-picryl hydrazyl(DPPH), ferric reducing antioxidant power (FRAP), N, N-dimethyl-p-phenylenediamine (DMPD) and 2,2-azino-bis (3-ethylbenzo-thiazoline-6-sulphonic acid) (ABTS). The antioxidant capacity for DPPH was recorded as % RSA, while for FRAP, DMPD and ABTS assays was calculated as µM ascorbic acid equivalent antioxidant capacity (AEAC).

DPPH radical scavenging assay of the plant extracts were assessed as a degree of radical scavengers using 1, 1-diphenyl-2-picrylhydrazyl in 100 ml of chilled methanol (Brand-Williams et al. 1995). 100 µl of plant extract were reacted with 2.9 ml DPPH solution. The reaction suspension was shaken and kept in dark for 35 min after which the absorbance was measured at 517 nm.

An improved decolorization assay N, N-dimethyl-p-phenylenediamine (DMPD) was performed to evaluate

antioxidant capacity (Fogliano et al. 1999). A mixture of extract (150 µl) and distilled water (0.850 ml) were allowed to react with 2 ml of DMPD reagent. After 15 min dark incubation the absorbance were measured at 505 nm.

The FRAP assay was demonstrated using the procedure described by Benzie and Strain (1996). The absorbance was read at 595 nm after 15 min dark incubation at 37 °C.

The ABTS assay was carried out according to method given by Subramanya et al. (2015) with minor modifications. ABTS reagent formed by assimilation of 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution as equal amount and kept in dark for 12 h. Incubated reagent diluted in methanol (1.1 ± 0.02 absorbance at 734 nm) which gives ABTS reagent for reaction. The plant extracts (0.1 ml) were allowed to react with 2.9 ml of the ABTS reagent, the reaction mixture was kept in dark for 2 h and the absorbance was recorded at 734 nm.

Optimization of cell suspension cultures and elicitor's treatments

2.0 g of fragile callus produced on MS medium fortified with 2, 4-D (2.0 mg/l) were placed in 250 ml conical flasks containing 100 ml of liquid growth medium. Samples were kept in at 25 ± 2 °C for 16/8 h (light/dark) over 4 weeks (taken to sub-culture at every 2 weeks) on the orbital shaker with an average speed of 100 rpm. The fresh and dry weight were recorded by harvesting the cells to different day's intervals.

The elicitors *viz.* methyl jasmonate (MeJA), salicylic acid (SA) at micromolar (µM) and chitosan (CH) at parts per million (ppm), which activate the cell's defense systems and triggers active principle metabolites in cell suspension cultures were applied in different concentrations to 25, 50, 100 and 200. Cell suspension cultures established in liquid MS medium fortified with 2,4-D (2.0 mg/l) and NAA (1.0 mg/l) treated as control. Then, cell suspension cultures were kept in 16/8 h light/dark conditions at 25 ± 2 °C in the climate chamber of orbital shaker with an average speed of 100 rpm. The effects of different concentrations of the elicitors were recorded by collecting the cell biomass after 25 days of treatment duration and used for quantification of swertiamarin in methanolic, ethanolic and water extracts prepared using dried cell powder.

Quantitative determination of swertiamarin in elicitor-treated cell suspension cultures

Extract and standard preparation

Extracts were prepared with 100 mg plants powder in 10 ml of respective solvent (methanol, ethanol and water) for 24 h on orbital shaker. HPLC grade swertiamarin was procured from ChromaDex (99.3% pure; ChromaDex, USA). An

accurately weighed standard swertiamarin was dissolved in known amount of methanol to obtain 1 mg/ml concentration of stock. The stock solution was then diluted to obtain desired working concentrations (1, 5, 10, 20, 40, 80, and 100 µg/ml).

Reverse phase-ultra flow liquid chromatographic analysis

The RP-UFLC analysis was performed on Shimadzu chromatographic system consisting of a quaternary pump, manual injector and dual λ UV absorbance diode array detector. For data processing, the built in LC-Solution software system was used. Chromatographic separation was done on a Lichrospher 100, C18e (5 µm) column (250–4.6 mm). Mobile phase consisting of acetonitrile and water (25:75) was used for separation with injection volume 10 µL. The flow rate was 1 ml/min and the detection wavelength of the dual λ absorbance detector beam was set at 238 nm. The analysis time was 5 min for both standard and sample. The system suitability was assessed by injecting three replicate of the standard solution.

Statistical analysis

Plant tissue culture experiments were conducted in randomized block design with 20 replicates per treatment and the experiments were repeated thrice. The results were expressed as mean \pm standard error (SE). The values are significantly different at ns-non significant, * $P < 0.05$ and ** $P < 0.01$ level as compared by Dunnett multiple comparisons test. Phytochemical experiments were repeated thrice and results were calculated as average \pm standard error (SE). A dendrogram was constructed by using Bray-Curtis Cluster Analysis software based on RAPD results.

Results and discussion

In vitro regeneration progression

The seeds of *S. minor* are very minute hence the surface-sterilized fruits were opened using a sterile surgical blade and seeds were allowed to set on sterilized wet cotton moistened with MS liquid medium in cultures tubes. Seed germination was observed after 1 week of incubation (Fig. 1a). The cultures were established using shoot tips of well-developed seedlings on MS medium supplemented with BAP (1.0 mg/l) (Fig. 1b). In vitro seed germination approach has previously been successfully employed for production of genetically stable plantlets of *S. lawii* (Kshirsagar et al. 2015b). In vitro shoot production was strongly influenced by the type, concentration and combinations of plant growth regulators used (Table 1).

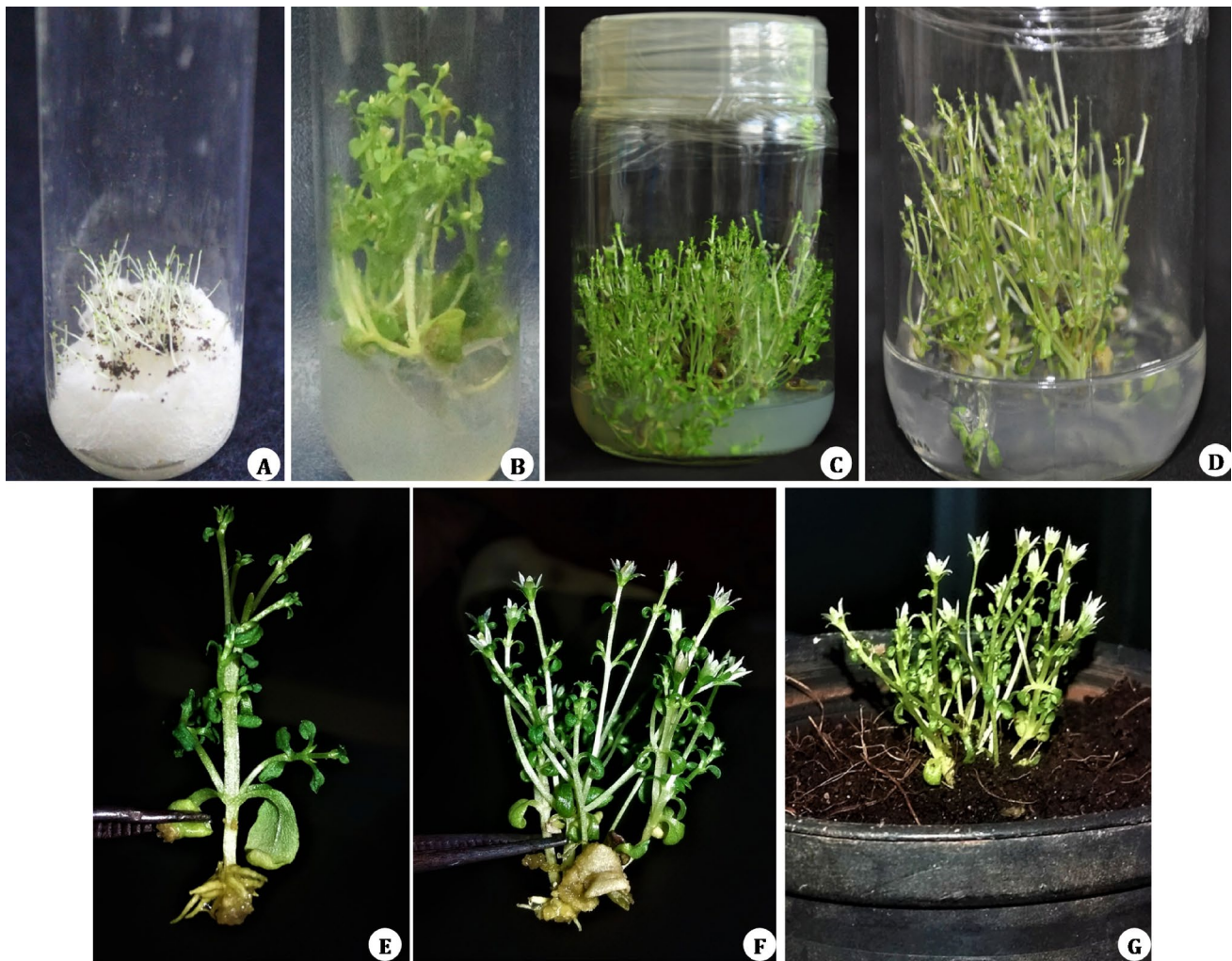


Fig. 1 Direct shoot organogenesis, in vitro rooting and acclimatization of *S. minor*: **a** In vitro seed germination; **b** shoot induction and multiplication on MS+BAP (1.0 mg/l); **c**, **d** shoot proliferation on

MS+BAP (3.0 mg/l)+TDZ (1.0 mg/l); **e** rooting on MS media with IBA (2.0 mg/l); **f** root induction in MS media with NAA (1.0 mg/l); **g** hardened plant

MS medium devoid of plant growth regulators failed to induce the shoots (Table 1). The shoots were initiated after 10 days of culture. The shoots were multiplied by repetitive transfer of the original explants on the fresh medium after harvesting freshly induced shoot tips. Of the PGRs tested, the numbers of shoots produced were significantly better in TDZ treatments than those recorded for BAP. However, individual supplementation of BAP supported the enhanced shoot length in *S. minor*. A maximum of 19.1 ± 0.95 shoots was produced when nodal explants were shifted to the fresh MS medium supplemented with a mixture of BAP (3.0 mg/l) and TDZ (1.0 mg/l) (Table 1; Fig. 1c, d). Likewise, a mixture of cytokinins were proved better for shoot production in *Swertia chirata* (Balaraju et al. 2009) and *Ceropegia spiralis* (Chavan et al. 2011). Recently, Saha et al. (2018) reported the enhanced shoot production of *S. chirayita* using silver nanoparticles.

The current study demonstrated the effects of different PGRs and their combinations on callus induction, indirect shoot organogenesis and embryogenesis processes of *S. minor* (Table 2). The calli showed varied responses according to type, concentration and combination of exogenous PGRs (Table 2). Various concentrations of 2,4-D and NAA supported the callus induction. Callogenesis occurred on leaf explants of the in vitro germinated seedling explants of *S. minor* after 7 days of incubation period. Among auxin tested, the superior response was observed when explant transferred to 2,4-D supplemented MS medium. The best callus formation with 95% callus induction frequency was obtained on MS medium supplemented with 2,4-D (2.0 mg/l). This combination resulted in production of pale yellow, watery and fragile callus (Fig. 2a). However, greenish friable to compact calli were produced in response to NAA treatments. Previously, best callogenic

Table 1 Effect of various concentrations of plant growth regulators on direct shoot organogenesis in *S. minor*

Sr. No.	PGRs	Concentration (mg/l)	Regeneration frequency (%)	No. of days required for shoot initiation \pm SD	Average no. of shoots \pm SD	Average shoot length (cm) \pm SD
1.	PGR free		00	00	00	00
2.	BAP	1.0	85**	5.1 \pm 0.2**	7.2 \pm 0.36**	4.3 \pm 0.21**
3.		2.0	90**	6.3 \pm 1.0**	7.6 \pm 0.38**	4.7 \pm 0.18**
4.		3.0	95**	6.0 \pm 0.5**	8.2 \pm 0.41**	5.6 \pm 0.22**
5.		4.0	75**	6.1 \pm 0.8**	6.2 \pm 0.31**	4.1 \pm 0.16**
6.	TDZ	1.0	85**	11.6 \pm 1.1**	12.6 \pm 0.63**	3.0 \pm 0.12**
7.		2.0	80**	10.8 \pm 1.0**	13.4 \pm 0.67**	3.2 \pm 0.12**
8.		3.0	80**	11.1 \pm 0.4**	9.5 \pm 0.47**	2.7 \pm 0.10*
9.		4.0	70**	11.1 \pm 0.9**	10.2 \pm 0.51**	2.8 \pm 0.11**
10.	BAP+TDZ	3.0+0.5	80**	9.0 \pm 1.3**	12.5 \pm 0.62**	2.6 \pm 0.10*
11.		3.0+1.0	85**	9.6 \pm 0.3**	19.1 \pm 0.95**	2.7 \pm 0.10*
12.		3.0+2.0	80**	9.2 \pm 0.4**	14.4 \pm 0.72**	3.0 \pm 0.12**
13.		3.0+3.0	80**	9.6 \pm 0.8**	13.6 \pm 0.68**	2.6 \pm 0.10*

Mean \pm S.E. of 20 replicates per treatment

The values are significantly different at *P<0.05 and **P<0.01 level as compared by Dunnett multiple comparisons test

Table 2 Effect of various concentrations of different PGRs on differential responses of callus in *S. minor*

Sr. No.	PGRs	Conc. (mg/l)	RF (%)	No of Shoots \pm SD	Remark
1	PGR free				
2	2,4-D	1.0	85	–	Creamy
3		2.0	95	–	Creamy, fast growing
4		3.0	80	–	Compact
5		4.0	70	–	Compact
6		5.0	65	–	Compact
7	NAA	1.0	75	–	Creamy
8		2.0	70	–	Compact calli, embryonic
9		3.0	70	–	Compact calli, embryonic
10		4.0	65	–	Compact, embryonic
11		5.0	65	–	Compact
12	2,4-D+NAA	1.0+0.5	80	–	Globular appearances
13		1.0+1.0	95	–	Calli with globular embryos
14		1.0+1.5	90	–	Late embryonic stage
15		1.0+2.0	80	–	Torpedo
16		2.0+0.5	85	–	Torpedo
17		2.0+1.0	95	–	Fast growth, loose (used for suspension studies)
18		2.0+1.5	90	–	Loose, creamy, greenish
19		2.0+2.0	85	–	Greenish, compact
20	2,4-D+BAP	2.0+1.0	80	–	Emergence of shoot primordia
21		2.0+2.0	80	3.1 \pm 0.3	Slow growth of shoot primordia
22		2.0+3.0	75	5.6 \pm 0.3	Slow growth of shoot primordia
23		2.0+4.0	70	6.1 \pm 1.0	Well-developed shoots from calli
24		2.0+5.0	70	6.0 \pm 0.6	Shoot development

responses have been observed on singular supplementation of 2,4-D in *S. lawii* (Kshirsagar et al. 2015b) and *S. chirayita* (Kumar et al. 2018), which are in accordance with

our results. In contrast, 2,4-D in combination with BAP promoted the in vitro callus induction from root-explant of *S. chirayita* (Pant et al. 2012).

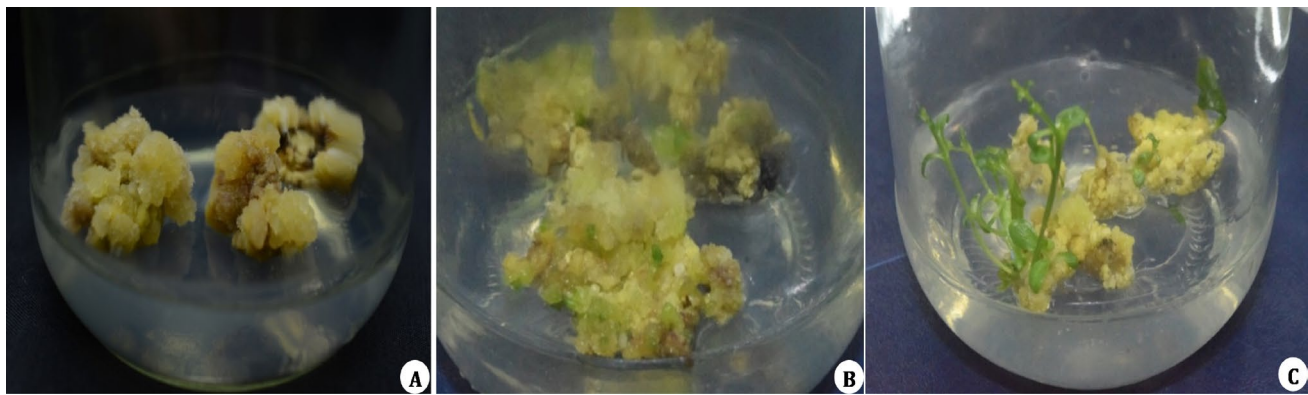


Fig. 2 Differential responses shown by callus cultures of *S. minor*. **a** Callus induction and proliferation on MS + 2,4-D (2.0 mg/l); **b** emergence of shoot primordia on MS + 2,4-D (2.0 mg/l) + BAP (1.0 mg/l); **c** shoot development on MS + 2,4-D (2.0 mg/l) + BAP (4.0 mg/l).

In the current study, a stable supplementation of 2,4-D (2.0 mg/l) along with varied concentrations of BAP (1.0–5.0 mg/l) promoted the shoot initiation from callus. It was noticed that, lower concentrations of BAP induced the shoot primordial, while increasing concentrations resulted in maturation and development of shoots (Fig. 2b). Maximum number of shoots (5.8 ± 0.3) were recorded when callus was transferred to MS medium fortified with 2,4-D (2.0 mg/l) in combination with BAP (3.0 mg/l) with 75% of regeneration frequency (Fig. 2c). In contrast, either singular supplementation of BAP or its combination of GA₃ supported the induction of shoots from calli of *S. chirayita* (Kumar and Chandra 2013). In further studies on *S. chirayita* Kumar et al. (2014), it was reported that a combination of BAP, adenine sulphate and IAA resulted in production of maximum number of shoots from calli of *S. minor*.

The well-growing friable callus obtained from 2,4-D (2.0 mg/l) treatment were transferred to mixture of auxins for testing their effects on induction of somatic embryos (Table 2). Lower concentration of 2,4-D (1.0 mg/l) along with NAA (0.5 mg/l) resulted in formation of globular clumps of somatic embryos (Fig. S1A). Slight enhancement in the concentrations of NAA (2.0 mg/l) resulted in the production of heart and torpedo shaped somatic embryos in *S. minor* (Fig. S1B, C). Previously, a combination of 2,4-D and kinetin triggered the induction of somatic embryos in *S. chirayita* (Jha et al. 2011). However, direct somatic embryogenesis approach is the most well-known mode to induce embryos and subsequent development of complete plantlets (Balaraju et al. 2011; Mahendran and Narmatha Bai 2017). In the present study, a very low frequency noticed for transformation of embryos into whole plant. Similarly, induction of somatic embryos was restricted to few stages has been reported for four *Ceropegia* species (Chavan et al. 2018).

The well-developed shoots with 4–5 pairs of leaves were transferred to MS medium supplemented with different types and concentrations of plant growth regulators for *in vitro*

rooting (Table 3). MS medium without PGRs was incapable of inducing roots in *S. minor*. The supplementation of MS media with IBA (2.0 mg/l) was found to be best (9.2 ± 0.56) for rooting of the *in vitro* shoots in *S. minor* with 90% root induction frequency (Table 3; Fig. 1e). In the same media composition, the roots were thick, hairy and healthy. In contrast, NAA (1.0 mg/l) resulted in stunted growth as well as slight reduced number of roots (6.6 ± 0.36) as compared to IBA treatments (Fig. 1f); however, the media combination supported shoot multiplication along with formation of roots. Such healthy growing shoots provides added advantage during hardening and acclimatization of *S. minor*. The action of auxins on root production is ascribed to a

Table 3 Effect of different auxins on *in vitro* rooting of *S. minor*

PGRs	Concentration (mg/l)	Regeneration frequency (%)	Average number of roots \pm SD	Average length of roots (cm) \pm SD
PGR free	00	00	00	00
IBA	1.0	80	$6.1 \pm 0.46^{**}$	$1.1 \pm 0.05^*$
	2.0	90	$9.2 \pm 0.56^{**}$	$1.9 \pm 0.06^{**}$
	3.0	75	$7.5 \pm 0.32^{**}$	$1.5 \pm 0.02^{**}$
	4.0	75	$7.5 \pm 0.37^{**}$	0.4 ± 0.02^{ns}
IAA	1.0	90	$7.2 \pm 0.41^{**}$	$1.0 \pm 0.05^*$
	2.0	90	$7.4 \pm 0.37^{**}$	$1.1 \pm 0.05^*$
	3.0	80	$6.4 \pm 0.32^{**}$	0.5 ± 0.02^{ns}
	4.0	80	$6.2 \pm 0.31^{**}$	$1.0 \pm 0.05^*$
NAA	1.0	80	$6.6 \pm 0.36^{**}$	0.5 ± 0.07^{ns}
	2.0	90	$5.5 \pm 0.27^{**}$	0.9 ± 0.05^{ns}
	3.0	70	$7.1 \pm 0.35^{**}$	0.8 ± 0.04^{ns}
	4.0	70	$6.2 \pm 0.30^{**}$	0.5 ± 0.02^{ns}

Mean \pm S.E. of 20 replicates per treatment

The values are significantly different at *ns* non significant, * $P < 0.05$ and ** $P < 0.01$ level as compared by Dunnett multiple comparisons test

few factors, including its preferential uptake, transport and metabolism (Ludwig-Muller 2000).

The ability of regenerates to survive under field conditions is of great significance as it determines the success of large-scale *in vitro* propagation. Plantlets have to undergo a transitional phase from the protective, mild optimal environment *in vitro* to an exposed environment in the field that can be detrimental to their survival. In this study, the rooted plantlets were successfully transferred into plastic cups containing a sterile sand, soil and coco peat (1:1:1), irrigated with ½-strength MS basal medium without sucrose and pots were covered with polythene bags. The survival rate of plantlets was 78% (Fig. 1c). The plants undergo normal growth and no visible morphological differences occur when compared with the mother stock.

Molecular profile of micropropagated clones

The main objective of *in vitro* regeneration is to produce genetic clone of the true-to-type plants. In contrast, the plantlets derived through indirect organogenesis shows slight divergence in genetic composition. The DNA based RAPD marker method are highly significant to declare the genetic fidelity of micropropagated plants. Out of 25 RAPD primers screened, 18 primers gave clear and reproducible bands with the mother as well as micropropagated plants. Overall, 983 bands were regenerated from a micropropagated plantlets

and mother plant amplified with 18 RAPD primers, out of that 87 bands are monomorphic and 39 bands are polymorphic (Table 4). The results of present study determined that, micropropagated copies of DNA (well no 1 to well no 10) displayed higher monomorphism with less than 30% of polymorphism (Fig. 3a, b). Dendrogram obtained also suggested the maximum similarity among regenerants of *S. minor* (Fig. 3c). Similarly, in our previous studies RAPD markers were found effective for examination the genetic stability of *S. lawii* (Kshirsagar et al. 2015b). Joshi and Dhawan (2007) suggested the utility of ISSR markers for genetic stability evaluation of micropropagated *S. chirayita*; however, in another study a combined approach of RAPD and ISSR was found effective for regenerants of *S. chirayita* (Sharma et al. 2016). Such combined use of RAPD and ISSR were also reported in other medicinal plants including *Ceropegia evansii* (Chavan et al. 2015).

Polyphenolic profile

Investigation of chemical makeup of *S. minoris* of much importance in order to establish a relationship and understanding its immense medicinal attributes. The present study tested the total phenolics and flavonoids content in wild grown plants, callus and *in vitro* derived plants revealed a significant amount of polyphenols (Table 5). The values of TPC was ranged from 6.50 ± 0.07 to 20.49 ± 0.10 mg GAE/g

Table 4 List of primers, their sequences and size of the amplified products generated by RAPD primers

S. no	Primer code	Primer sequence (5'–3')	Number of allele/primer	No. of mono-morphic bands	No. of poly-morphic bands	Total number of amplified bands/primer	Size range (bp)
1	RPI 1	AAAGCTGCGG	9	7	2	77	200–1500
2	RPI 3	AGTCAGCCAC	6	4	2	47	400–1500
3	RPI 4	AATCGCGCTG	7	5	2	57	400–1500
4	RPI 6	ACACACGCTG	5	2	3	28	600–1500
5	RPI 7	ACATCGCCCA	6	5	1	46	100–1500
6	RPI 8	ACCACCCACC	4	4	0	35	300–1000
7	RPI 10	ACGATGAGCG	9	6	3	67	200–1500
8	RPI 11	ACGGAAGTGG	8	7	1	70	300–1500
9	RPI 13	ACGGCAAGGA	8	5	3	58	200–900
10	RPI 16	AGGCGGCAAG	6	4	2	44	300–1500
11	RPI 17	AGGCGGGAAC	9	5	4	62	200–1500
12	RPI 18	AGGCTGTGTC	7	5	2	56	200–3000
13	RPI 19	AGGTGACCGT	7	5	2	57	200–3000
14	RPI 21	CACGAACCTC	11	4	7	70	100–1500
15	RPI 22	CATAGAGCGG	6	6	0	58	200–1500
16	RPI 23	CCAGCAGCTA	5	4	1	41	300–3000
17	RPI 24	CCAGCCGAAC	7	5	2	61	300–3000
18	RPI 25	GAGCGCCTTC	6	4	2	49	400–1500
			126	87	39	983	

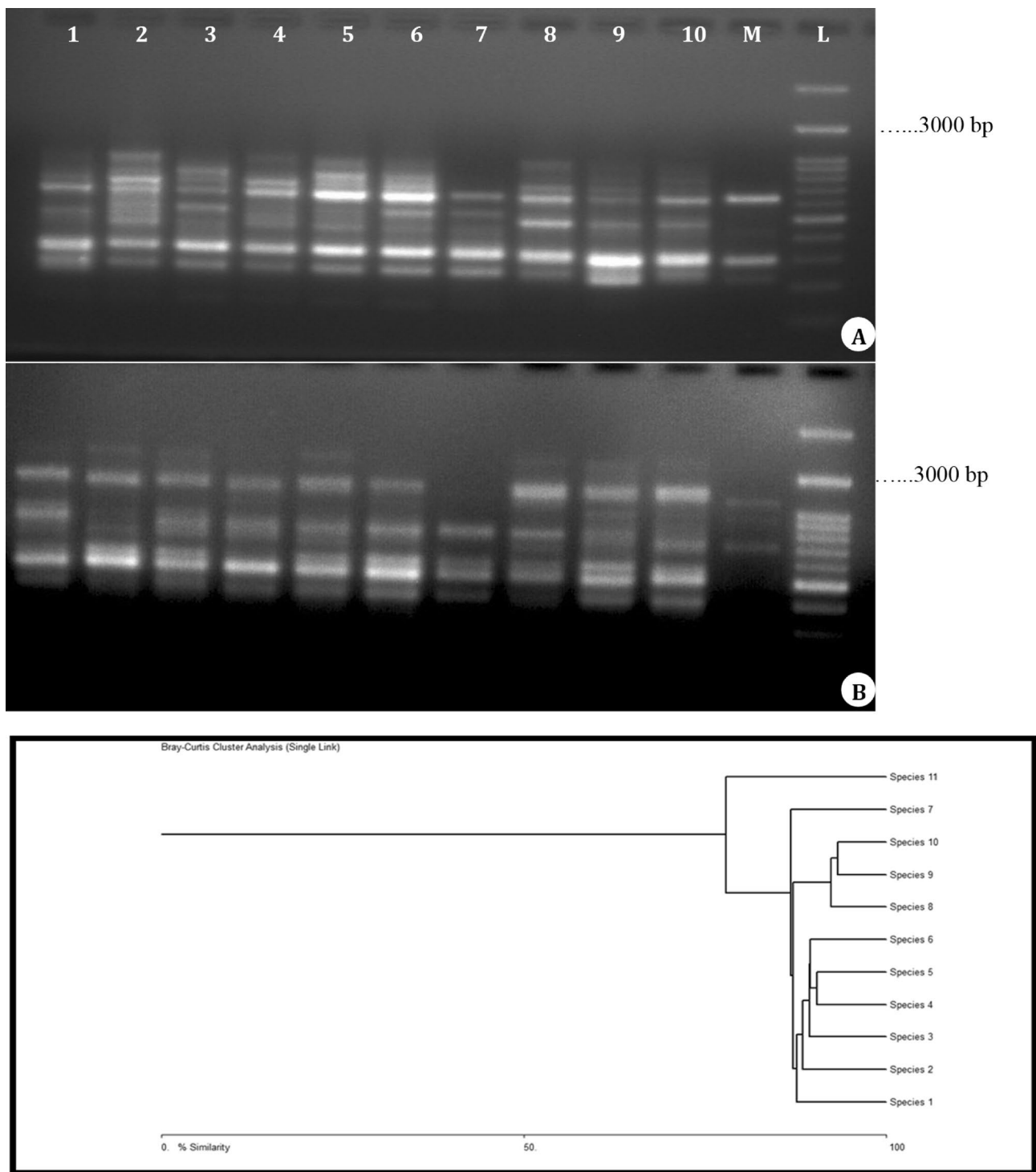


Fig. 3 DNA fingerprinting pattern generated with RAPD primer. **a** RPI-4, **b** RPI-23, **c** dendrogram obtained Bray-Curtis Cluster Analysis. Lane 1–10: in vitro raised clones; Lane 11: Mother Plant; Lane L: molecular weight marker.

DW and 6.96 ± 0.07 to 22.18 ± 0.10 TAE/g DW. Continuous shaking extraction resulted in extraction of higher amounts total phenolics in ethanol as compared to other extraction modes and solvent systems used. The results of the present

study indicated that among the samples evaluated, ethanolic extract of wild grown plants found to possess higher values for phenolic contents (20.49 ± 0.10 mg GAE/g DW and 22.18 ± 0.10 TAE/g DW) followed by in vitro derived

Table 5 Comparative profile of total phenolics and flavonoids content among wild grown, callus and *in vitro* grown plants of *S. minor*

Samples	Method	Solvent	TPC mg/g		TFC mg/g	
			GAE	TAE	RE	QE
<i>S. minor</i> (wild)	SE	Methanol	16.31 ± 0.08	17.63 ± 0.08	85.25 ± 0.06	72.68 ± 0.06
		Ethanol	15.23 ± 0.10	16.46 ± 0.10	85.42 ± 0.09	72.81 ± 0.09
		Water	16.63 ± 0.11	17.98 ± 0.11	81.35 ± 0.07	69.64 ± 0.07
	CSE	Methanol	19.48 ± 0.07	21.08 ± 0.07	114.82 ± 0.07	95.78 ± 0.07
		Ethanol	20.49 ± 0.10	22.18 ± 0.10	129.75 ± 0.08	107.45 ± 0.08
		Water	18.99 ± 0.07	20.55 ± 0.07	92.82 ± 0.07	78.59 ± 0.07
	UE	Methanol	19.22 ± 0.09	20.79 ± 0.09	111.69 ± 0.09	93.33 ± 0.09
		Ethanol	14.22 ± 0.10	15.36 ± 0.10	71.05 ± 0.07	61.59 ± 0.07
		Water	17.95 ± 0.12	19.41 ± 0.12	86.22 ± 0.11	73.44 ± 0.11
<i>S. minor</i> (Callus)	SE	Methanol	10.65 ± 0.09	11.48 ± 0.09	40.62 ± 0.07	37.81 ± 0.07
		Ethanol	10.34 ± 0.09	11.14 ± 0.09	42.29 ± 0.10	39.11 ± 0.10
		Water	06.50 ± 0.07	06.96 ± 0.07	05.55 ± 0.08	10.42 ± 0.08
	CSE	Methanol	13.28 ± 0.08	14.33 ± 0.08	46.89 ± 0.10	42.71 ± 0.10
		Ethanol	13.39 ± 0.07	14.46 ± 0.07	54.45 ± 0.11	48.62 ± 0.11
		Water	06.74 ± 0.08	07.22 ± 0.08	06.62 ± 0.07	11.25 ± 0.07
	UE	Methanol	14.00 ± 0.08	15.12 ± 0.08	50.52 ± 0.06	45.55 ± 0.06
		Ethanol	08.34 ± 0.11	08.96 ± 0.11	32.32 ± 0.09	31.33 ± 0.09
		Water	06.70 ± 0.10	07.17 ± 0.10	06.95 ± 0.09	11.51 ± 0.09
<i>S. minor</i> (In vitro)	SE	Methanol	12.50 ± 0.10	13.49 ± 0.10	65.15 ± 0.10	56.98 ± 0.10
		Ethanol	09.48 ± 0.08	10.20 ± 0.08	52.85 ± 0.09	47.37 ± 0.09
		Water	13.33 ± 0.08	14.39 ± 0.08	35.32 ± 0.09	33.67 ± 0.09
	CSE	Methanol	15.93 ± 0.09	17.22 ± 0.09	81.75 ± 0.08	69.95 ± 0.08
		Ethanol	13.66 ± 0.08	14.75 ± 0.08	85.72 ± 0.07	73.05 ± 0.07
		Water	12.41 ± 0.11	13.38 ± 0.11	34.99 ± 0.09	33.41 ± 0.09
	UE	Methanol	13.98 ± 0.09	15.09 ± 0.09	68.12 ± 0.09	59.30 ± 0.09
		Ethanol	08.34 ± 0.10	08.96 ± 0.10	42.62 ± 0.07	39.38 ± 0.07
		Water	13.78 ± 0.09	14.88 ± 0.09	37.15 ± 0.10	35.10 ± 0.10

plants and callus respectively. However, not much variation in TPC content was observed among extraction techniques, solvent systems and the samples. Maximum values of 20.49 ± 0.10 mg GAE/g DW and 22.18 ± 0.10 TAE/g DW were recorded for wild grown plants when extracted using continuous shaking extraction procedure and ethanol as solvent system (Table 5). Callus extract in water using static extraction procedure resulted in lowest values for total phenolics (6.50 ± 0.07 mg GAE/g DW and 6.96 ± 0.07 TAE/g DW). A similar trend was observed during extraction of total flavonoids content; however, the mode of extraction, solvent system and samples used significantly altered the values of TFC (Table 5). The amounts of TFC was ranged from 5.55 ± 0.08 to 129.75 ± 0.08 mg RE/g DW to 10.42 ± 0.08 to 107.45 ± 0.08 mg QE/g DW. Higher value of TFC was recorded in wild grown plants when extracted through continuous shaking extraction in ethanol, whereas callus extract in water showed poor extraction of TFC (Table 5). The poor values for TFC might be due to the formation of complex polymerized substances, which are more extractable in ethanol and methanol than water extracts. Our findings have

revealed that the wild grown *S. minor* plants possessed significant contents of total phenolics as well as flavonoids as compared to callus and in vitro derived plants. Our results are in line with the previous studies addressed the higher contents of total phenolics and flavonoids in wild grown plants as compared to in vitro raised plants of *Swertia corymbosa* (Mahendran and Narmatha Bai 2014).

Antioxidant properties

In the present investigation, four in vitro tests (DPPH, FRAP, DMPD and ABTS) based on various reaction mechanisms were performed to evaluate the antioxidant potential of in vitro propagated plants, callus and this was compared with level of wild grown plants (Table 6). Very little dissimilarities were observed for antioxidant properties for *S. minor* and it was noticed that the activity was not found to be strictly dependent of samples, extraction mode and solvent system used (Table 6). There were only small differences in the antioxidant properties due to the samples, extraction mode and solvent system. The antioxidant potential was recorded

Table 6 Antioxidant potential of wild grown, in vitro grown plants and callus of *S. minor*

Samples	Method	Solvent	DPPH % RSA	DMPD (μM AEAC)	FRAP (μM AEAC)	ABTS (μM AEAC)
<i>S. minor</i> (wild)	SE	Methanol	58.28 \pm 0.06	601.11 \pm 0.07	1066.33 \pm 0.06	684.44 \pm 0.07
		Ethanol	54.00 \pm 0.07	623.33 \pm 0.06	1085.67 \pm 0.06	773.33 \pm 0.07
		Water	64.41 \pm 0.07	486.67 \pm 0.07	1091.00 \pm 0.07	637.78 \pm 0.06
	CSE	Methanol	68.89 \pm 0.04	593.33 \pm 0.08	1131.00 \pm 0.05	585.56 \pm 0.07
		Ethanol	76.87 \pm 0.06	613.33 \pm 0.06	1240.00 \pm 0.07	521.11 \pm 0.07
		Water	74.37 \pm 0.06	486.67 \pm 0.06	1145.67 \pm 0.05	601.11 \pm 0.06
	UE	Methanol	77.28 \pm 0.07	582.78 \pm 0.06	1165.33 \pm 0.08	655.56 \pm 0.07
		Ethanol	55.37 \pm 0.10	601.67 \pm 0.06	968.67 \pm 0.08	887.78 \pm 0.07
		Water	71.25 \pm 0.09	500.56 \pm 0.05	1116.00 \pm 0.06	632.22 \pm 0.06
<i>S. minor</i> (Callus)	SE	Methanol	25.36 \pm 0.07	647.78 \pm 0.08	554.67 \pm 0.05	812.22 \pm 0.07
		Ethanol	20.36 \pm 0.06	709.44 \pm 0.06	557.00 \pm 0.07	875.56 \pm 0.07
		Water	13.21 \pm 0.08	612.78 \pm 0.06	174.33 \pm 0.06	982.22 \pm 0.08
	CSE	Methanol	29.33 \pm 0.07	624.44 \pm 0.05	614.67 \pm 0.07	854.44 \pm 0.06
		Ethanol	25.94 \pm 0.07	703.89 \pm 0.07	727.67 \pm 0.06	792.22 \pm 0.06
		Water	14.13 \pm 0.07	633.89 \pm 0.06	173.00 \pm 0.08	980.00 \pm 0.05
	UE	Methanol	31.62 \pm 0.07	516.67 \pm 0.07	677.00 \pm 0.07	768.89 \pm 0.06
		Ethanol	19.10 \pm 0.05	618.89 \pm 0.06	375.67 \pm 0.08	970.00 \pm 0.05
		Water	12.83 \pm 0.07	471.11 \pm 0.07	219.33 \pm 0.08	770.00 \pm 0.04
<i>S. minor</i> (In vitro)	SE	Methanol	24.85 \pm 0.07	593.89 \pm 0.07	594.33 \pm 0.08	832.22 \pm 0.06
		Ethanol	18.07 \pm 0.07	657.22 \pm 0.07	559.67 \pm 0.05	912.22 \pm 0.09
		Water	34.80 \pm 0.06	312.22 \pm 0.09	569.67 \pm 0.06	775.56 \pm 0.08
	CSE	Methanol	34.39 \pm 0.06	547.78 \pm 0.08	781.33 \pm 0.06	751.11 \pm 0.07
		Ethanol	29.26 \pm 0.08	598.33 \pm 0.05	793.33 \pm 0.08	774.44 \pm 0.05
		Water	35.32 \pm 0.06	346.11 \pm 0.06	167.67 \pm 0.05	803.33 \pm 0.05
	UE	Methanol	31.28 \pm 0.07	550.56 \pm 0.06	677.33 \pm 0.08	914.44 \pm 0.05
		Ethanol	14.89 \pm 0.08	679.44 \pm 0.07	456.00 \pm 0.06	926.67 \pm 0.03
		Water	41.62 \pm 0.08	316.67 \pm 0.07	566.00 \pm 0.07	816.67 \pm 0.06

highest in each assay were as follows, for DPPH assay (77.28 \pm 0.07%, in methanolic extract of wild grown plants), for DMPD assay (709.44 \pm 0.06 μM AAE, in ethanolic callus extract), for FRAP assay (1240.00 \pm 0.07 μM AAE, in ethanolic extract of wild grown plants) and for ABTS assay (982.22 \pm 0.08 AAE, in aqueous callus extract). The results obtained for DPPH and FRAP assays supports the previous reports where methanolic extract showed maximum antioxidant potential in *S. cordata* and *S. chirayita* (Roy et al. 2015). In current study, the lowest scavenging activity with 12.83 \pm 0.07% (in DPPH assay) and 173 \pm 0.08 μM ascorbic acid equivalent (FRAP assay) was demonstrated by the extract of callus in water and ethanol respectively (Table 6). In the present study, it was also observed that the aqueous extracts of wild grown, calli and in vitro- raised plants also showed significant values of antioxidant properties evaluated by all assays.

Growth kinetics of cell suspension cultures

Biomass formation of the cell suspension culture of *S. minor* displayed a relatively quick growth curve and was characterized by a lag phase of 4 days for fresh and dry biomass, followed by log phase of 15 days, and a subsequent stationary phase during 25-day period of study (Fig. 4). Above two-fold increase in two-fold increase in fresh weight (4.61 g) were recorded on 16th day of culture period. However, the fresh weight and dry weight displayed by cultures on 8th day were 4.20 g and 0.40 g, respectively (Fig. 4). Furthermore, cell suspension cultures were found to be milky white, yellow and brownish in colour during different growth phases respectively.

Swertiamarin content

Swertiamarin content in elicitor treated cell suspension cultures were determined by RP-UFLC. All treatments were found to contain swertiamarin accumulation with variable amounts and the values ranged from 0.12 to 1.45 mg/g DW

Fig. 4 Growth kinetics of cells in suspension cultures of *S. minor* on MS medium supplemented with 2,4-D (2.0 mg/l) and NAA (1.0 mg/l).

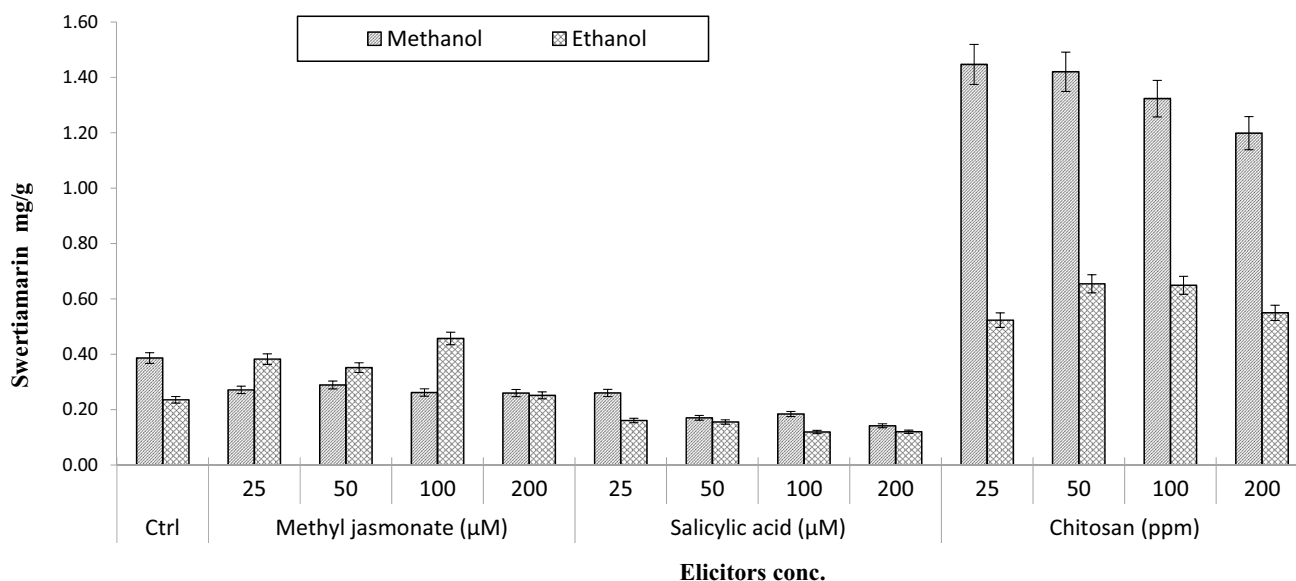
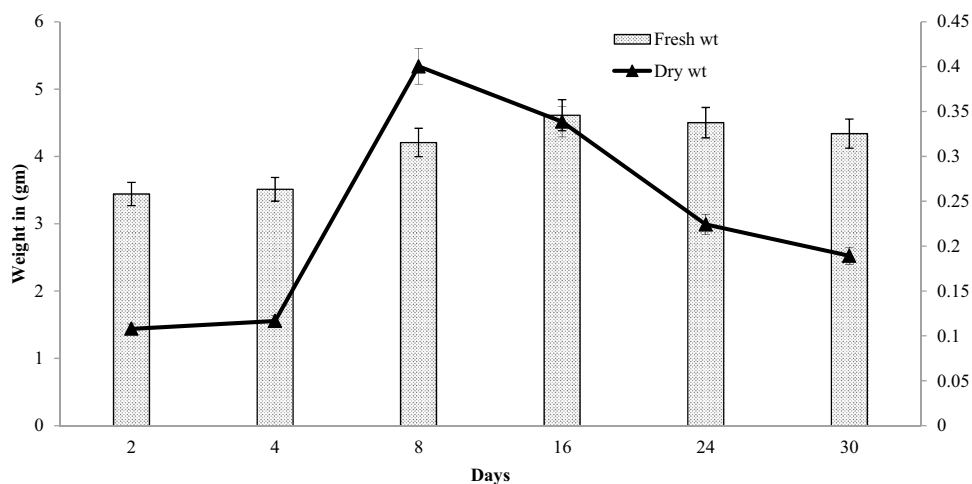


Fig. 5 Swertiamarin content in response to different solvents and elicitor treatments in cell suspension cultures of *S. minor*

(Fig. 5). Among the elicitors tested, chitosan (25 ppm) was found suitable for enhanced production of swertiamarin (1.45 mg/g) as compared to methyl jasmonate and salicylic acid (Fig. 5). The amount was 3.5 fold higher as compared to control treatment. Chitosan is the major component of exoskeletons of insects, crustacean and fungal cell wall which mimics the effects of some pathogen to stimulate plant secondary metabolites (Pliankong et al. 2018). Chitosan was also found beneficial for vincristine and vinblastine accumulation in cell culture of *Catharanthus roseus* (Pliankong et al. 2018). In the current study, the swertiamarin level in the salicylic acid treated cell lines was unstable and gradually decreased over time. Previous reports noticed the higher levels of swertiamarin in wild plants as compared to in vitro

cells, tissues and organ cultures (Mahendran and Narmatha Bai 2014) except *Swertia paniculata* (Kaur et al. 2020). The findings of present study resulted with higher accumulation of swertiamarin in elicitor treated cell suspension cultures of *S. minor* for first time.

Conclusions

In conclusion, a simple and cost-effective protocol has been developed for in vitro regeneration via direct organogenesis, callogenesis and indirect organogenesis for first time in *S. minor*. The tested plants (both in vitro derived and wild-grown) and calli showed good amount of polyphenol content

as well as showed significant antioxidant properties, however, the wild grown plants showed slight augmented values for polyphenols and antioxidant activity. Furthermore, 3.5-fold higher levels of swertiamarin production in chitosan treated cell suspension cultures. We believed that the present findings led to identification of the precise alternative to *S. chirayita* for its uses in pharmaceutical industry.

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Author contributions PRK, JJC, NBG designed the experiment, performed tissue culture experiments, contributed to writing and corrected manuscript. PRK, AM and SS performed phytochemical experiments, collected and analyzed data, wrote the manuscript. JJC, NBG and VAB helped in experimental accomplishment, corrected manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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