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Enhanced Somatic Embryogenesis and Viable Synthetic Seeds in *Picrorhiza scrophulariiflora* Pannell: A Conservation Milestone

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ABSTRACT

A novel protocol for somatic embryogenesis has been established using callus derived from various explants of *Picrorhiza scrophulariiflora*, which is highly valued and endangered due to its medicinal significance and is indigenous to the Eastern Himalayas. A wide range of callusing (8.11-100%) from different explants were noticed in WPM supplemented with either 2, 4-D or NAA alone (0.1-2.0 mg/l). The incorporation of Kn and BAP, at various concentrations alongside different levels of NAA did not enhance callus formation. Out of which, only the friable callus upon transfer to WPM medium with cytokinins such as Kn or BAP (0.1–2.0 mg/l), could produce embryogenic callus which later by supplementation with ABA (0.1-1.0 mg/l) for 2 weeks, produced 92% matured somatic embryos. Furthermore, in a medium containing WPM with Kn (0.5 mg/l) and GA_3 (0.5 mg/l), around 73% of the well matured somatic embryos of this species were germinated. However, leaf derived friable callus were, invariably, found to be superior in all aspects when compare with other explants. The encapsulated somatic embryos when stored at 4°C showed as high as 100% of germination when kept up to 15 days, which however, gradually decreased as the storage time increased and thus as high as 20% viability were recorded when kept for 105 days. Afterwards, plantlets derived from somatic embryos were successfully acclimatized and transplanted to the field. Random amplified polymorphic DNA analysis indicated the genetic uniformity of the donor plants, somatic embryo derived plantlets and the synthetic seed derived plantlets. This was the first report of somatic embryogenesis of this medicinally important genus.

Keywords Conservation, Medicinal plant, Somatic embryo, Genetic fidelity, Synthetic seed, DNA fingerprinting.

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INTRODUCTION

The genus *Picrorhiza* is categorized under the Plantaginaceae family (recently designated), formerly it was under Scrophulariaceae (Bhardwaj *et al.* 2021). *P. scrophulariiflora* Pennell is a medicinally valuable

plant at risk of extinction, primarily found in the limited habitat of sub-alpine and alpine regions of the eastern Himalayas (Bantawa *et al.* 2017). The rarity of this species stems from excessive harvesting for medicinal use, coupled with disorganized and unscientific collection methods (Bantawa 2017). Consequently, the wild population of this species has declined to a critical level, prompting recommendations in India for a ban on its collection from natural habitats (Nayer and Shastry 1990).

The chemical composition of the genus *Picrorhiza* has been extensively studied, particularly focusing on its two species, *P. kurroa* and *P. scrophulariiflora.* While both species are known for their abundance of iridoid glycosides, such as kutkoside and picroside-I, II and III, *P. scrophulariiflora* stands superior for containing additional plantamajoside as well as phenylethanoid glycosides, which are not present in *P. kurroa* (Bantawa 2017). Moreover, research by Bantawa *et al.* (2010) highlighted that *P. scrophulariiflora* possesses significantly higher levels of picroside I as well as picroside II compared to *P. kurroa* and identified elite clones within its populations. Consequently, not only does *P. scrophulariiflora* serve as a substitute for *P. kurroa*, but it also demonstrates superior chemical properties (Bantawa *et al.* 2017).

The rhizomes of *P. scrophulariiflora* are employed in both Chinese and Tibetan traditional medicine to treat numerous ailments, including fever, asthma, liver disorders and jaundice (Kafle *et al.* 2018). They also possess significant pharmaceutical properties, such as immunomodulatory, hepatoprotective and antiasthmatic activities (Bantawa 2017). Furthermore, no significant adverse effects have been documented for this plant (Almeleebia *et al.* 2022).

Numerous studies emphasize the importance of its conservation, cultivation and sustainable use (Bantawa *et al.* 2009a, 2009b, 2010, 2011a, 2011b, 2017, Bantawa 2017). To ensure the sustainable use and conservation of the elite germplasm of *P. scrophulariiflora*, we have outlined the first micropropagation protocol for this species using kinetin (Bantawa *et al*. 2010), as well as adenine sulfate and bavistin (Bantawa *et al.* 2009b). Additionally, our team was the first to report a plantlet regeneration protocol via callus (Bantawa *et al.* 2011a).

Tissue and cell culture play an important role in genetic improvement of plants. Somatic embryo being single cell origin is suitable explant for genetic manipulation of the plant. However, there is no report on the production of somatic embryo, synthetic seed production, the viability of synthetic seed and genetic fidelity of those plants which are derived from synthetic seed. Thus, this paper presents our findings on the production of somatic embryos, the development and viability of synthetic seeds derived from these embryos, and the genetic fidelity of the plants grown from synthetic seeds, which, to our knowledge, is being reported for the first time.

MATERIALS AND METHODS

Explant source

Earlier we have identified an elite genotype of *P. scrophulariiflora* through HPLC (high performance liquid chromatography), which contained a high amount of total picroside (Bantawa *et al.* 2010). From this same mother culture, we used the different explants in this present study for producing callus.

Induction of embryogenic callus

The callus was induced from different explants, such as leaf (1-2 cm), and nodal segment (each 1.5-2 cm), in Woody Plant Medium (WPM) (Lloyd and McCown 1980) following our earlier report (Bantawa *et al.* 2011a).

To induce and initiate somatic embryos, both friable as well as non-friable callus were transferred from callus induction medium to the media containing low doses of cytokinins such as Kn and BAP (0.1-2.0 mg/l) (Tables 1–2).

Maturation and germination

Callus that turned yellowish-green and green in color, and developed a nodular texture following proliferation, was sub-cultured into WPM containing various concentrations of ABA (abscisic acid) (Hi-media Chemicals Pvt Ltd India) at 0.1, 0.5 and 1.0 mg/l (Table 3). These cultures were then incubated for 2

Table 1. Induction of callus from various explant sources using different PGR combinations. Data (X) pooled at least from three independent experiments, ± = Standard Error, *= Rhizogenesis, **= Browning callus, ## = Friable callus, Ψ = Both friable and compact callus, # = Best combination, † = Compact callus.

PGRs	Concentration (mg/l)	Induction of embryogenic callus (%) after 8 weeks Explant source Leaf	Nodal segment	Shoot tip
0	0	0 r	0 r	0 r
2,4-D	0.1	11.0±0.44†^{p}	8.11±0.28†^{q}	49.22±0.88†^{l}
	0.5	100 *^{a}	69.22±0.54†^{i}	100*^{a}
	1.0	100 *^{a}	68.26±0.52*^{i}	100*^{a}
NAA	0.1	25.68±0.44## ^{n}	0 r	17.22±1.22##^{o}
	0.5	100##^{a}	74.56±0.96##^{h}	78.22±1.04 Ψ^{g}
	1.0	100##^{a}	78.26±0.98 Ψ^{g}	74.02±0.96Ψ^{h}
NAA + Kn	0.1+0.05	88.88±0.22 Ψ^{de}	8.12±0.88 Ψ^{q}	48.28±0.88 Ψ^{l}
	0.5+0.1	89.88±0.28**^{cd}	33.33±0.96 Ψ^{m}	68.89±0.96 Ψ^{i}
	1.0+0.1	88.08±0.84**^{e}	66.68±0.28 Ψ^{j}	32.38±1.16**^{m}
NAA + BAP	0.1+0.05	100 Ψ^{a}	58.89±1.22 Ψ^{k}	60.22±0.66 Ψ^{k}
	0.5+0.1	100 Ψ^{a}	82.00±0.88 Ψ^{f}	83.00±0.90 Ψ^{f}
	1.0+0.1	100**^{a}	92.28±1.02 Ψ^{b}	90.22±0.88 Ψ^{bc}

weeks. Subsequently, they were transferred to WPM without plant growth regulators (PGRs) and kept for another 6 weeks.

For the germination of the somatic embryos, various combinations of Kn (0.1, 0.5, 1.0 and 2.0 mg/l) with Gibberellic acid (GA_3) (Hi-media Chemicals Pvt Ltd, India) (0.5 mg/l) or BAP (0.1, 0.5, 1.0 and 2.0 mg/l) with GA_3 (0.5 mg/l) (Table 4) were tested in WPM and were incubated for 4 weeks.

Encapsulation of somatic embryo

Encapsulation of somatic embryo was done separately in distilled water and liquid WPM basal medium alone or in combination with 0.5 mg/l Kn and 0.5 mg/l GA_3 using 2, 3, 4, and 5% (w/v) sodium alginate gel (Sigma chemicals, USA). The pH of the medium was carefully set to 5.8. Calcium alginate beads were produced by encapsulation method (Kinoshita and Saito 1992). Different concentrations of $CaCl_2.2H_2O$ (Hi-media Chemicals Pvt Ltd, India) solutions (i.e., 3.6, 7.2, 10.8, and 14.7 g/l) were tested for 30 min in order to optimized the size, shape and texture of alginate beads for germination. Both the gel matrix and complexing agents were autoclaved at 15 psi pressure at 121ºC for 15 min. Encapsulation was achieved by blending the somatic embryos into the sodium alginate solution and then introducing them into the calcium chloride solution. Each drop contained only one somatic embryo. The beads holding the enclosed somatic embryos were collected and rinsed 2 to 3 times with sterile distilled water. Thus, the encapsulated somatic embryos were stored at 4 ºC for periods of 15, 30, 45, 60, 75, 90, and 105 days

Table 2. The effects of PGRs on the induction of somatic embryos from the callus of different explant sources. Data (X ± SE) pooled from three independent experiments.

PGRs	Concentration (mg/l)	Induction of somatic embryos (%) after 4 weeks Explant source Leaf	Nodal segment	Shoot tip
BAP	0	0o	0o	0o
	0.1	18±1.74^{l}	12±1.56^{n}	15±1.24^{m}
	0.5	30±1.44^{h}	25±0.84^{i}	21±1.22^{j}
	1.0	26±0.98i	22±1.60^{jk}	25±1.66^{i}
Kn	0.1	59±1.44^{c}	25±1.22^{i}	49±1.46^{e}
	0.5	**78±1.62^{a}**	**42±0.96^{f}**	**56±0.88^{d}**
	1.0	62±1.54^{b}	19±0.96^{k}	32±0.98^{g}

Table 3. The effect of ABA on the maturation of somatic embryos.

PGR	Concentration	Leaf	Nodal segments	Shoot tip
ABA	0.1	45.08±1.0^{f}	41.08±0.80^{g}	44.88±0.86^{f}
	0.5	79.29±0.46^{c}	58.66±0.64^{e}	65.25±1.06^{d}
	1.0	**92.26±0.98^{a}**	**79.54±0.28^{c}**	**80.29±0.62^{b}**

Table 4. The germination pattern among different explant derived somatic embryos. X data pooled from separate experiments; ± = Standard Error.

PGRs	Concentration (mg/l)	Germination of somatic embryos after 4 weeks		
		Leaf	Nodal segment	Shoot tip
KN +GA_3	0	12.81±0.22^{p}	0r	5.22±0.74^{q}
	0.1+0.5	65.22±0.48^{c}	24.26±0.62^{l}	38.22±0.60^{i}
	0.5+0.5	72.84 ±0.88^{a}	55.25±0.46^{e}	66.62±0.50^{b}
	1.0+0.5	25.66±0.26^{k}	45.52±0.22^{g}	58.68±0.55^{d}
BAP + GA_3	0.1+0.5	25.35±0.84^{kl}	22.28±0.42^{m}	20.66±0.82^{n}
	0.5+0.5	39.64 ±0.69^{h}	46.27±0.28^{f}	28.89±0.58^{j}
	1.0+0.5	20.24±0.98^{n}	29.28±0.66^{j}	14.22±0.86^{o}

(Table 5). The somatic embryos enclosed in the capsules were placed onto WPM solid medium devoid of plant growth regulators for cultivation.

Hardening and field transfer

Well rooted plantlets derived from both somatic embryos and encapsulated somatic embryos were taken to Kyoungnosla Nursery, Changu, Forest Department, Government of Sikkim, India, where they were transferred to plastic pots (6×8 cm) containing sterile forest black soil and sand (9:1). At this stage the transferred plantlets were kept at Hot house, maintaining high humidity (80% or above), for 8 weeks for acclimatization then after they were transferred to a larger plastic pot (8 × 10 cm) containing same potting mixture. During this time, the plants were kept outside the hot house under direct sun. Regular watering was done till the plants were fully grown.

Genetic fidelity

Genetic fidelity test was performed as per our earlier report (Bantawa *et al.* 2011a).

Data collection

The study was arranged following a CRD (Completely Randomized Design), and statistical analysis was performed using analysis of variance (ANOVA) to identify any significant variations among the means (Sokal and Rohlf 1995). To compare significantly different means, Duncan's multiple range test (DMRT) was conducted at a significance level of $p \leq 0.05$, utilizing Statistica software version 5.0 (INC StatSoft 1995).

Table 5. Effect of storage on the conversion of encapsulated shoot tips sased on nutrient composition in the encapsulating matrix.

Day of storage	Materials used to make sodium alginate beads and $CaCl_2$	(%) response	Number of shoots	Germinating days
15	Distilled water + Kn (0.5 mg/l)	0	0	NA
30	Distilled water + Kn (0.5 mg/l)	0	0	NA
45	Distilled water + Kn (0.5 mg/l)	0	0	NA
60	Distilled water + Kn (0.5 mg/l)	0	0	NA
75	Distilled water + Kn (0.5 mg/l)	0	0	NA
90	Distilled water + Kn (0.5 mg/l)	0	0	NA
105	Distilled water + Kn (0.5 mg/l)	0	0	NA
0	**WPM+Kn (0.5 mg/l)**	**100**	**Multiple**	10
15	WPM+Kn (0.5 mg/l)	100	Multiple	10
30	WPM+Kn (0.5 mg/l)	87	Multiple	12
45	WPM+Kn (0.5 mg/l)	74	Multiple	16
60	WPM+Kn (0.5 mg/l)	70	Single	18
75	WPM+Kn (0.5 mg/l)	58	Single	20
90	WPM+Kn (0.5 mg/l)	42	Single	20
105	WPM+Kn (0.5 mg/l)	20	Single	25

Effect of different PGRs on callus induction, somatic embryos formation, germination of somatic embryos and acclimatization as well as survival percentage during hardening were recorded on weekly basis. At least 25 cultures were taken in each treatment and data were pooled from three independent experiments. Standard errors (±SE) were also pooled from these data.

The effect of alginated beads were evaluated with respect to (i) Days taken for germination of encapsulated somatic embryos, (ii) Percent response of alginate beads with respect to germination and (iii) Number of shoots (i.e. single or multiple) formed per bead at the interval of 15 d for a period of 105 days. Conversion frequency was calculated on the basis of differentiation of shoots and roots. Each treatment was consisted of at least 20 beads per replication and all data were pooled from at least three independent experiments. Standard errors (±SE) were also pooled from these data.

RESULTS AND DISCUSSION

Explant source, stimulation of embryogenic callus formation and development of somatic embryo

To induce embryogenesis, both friable and compact non-friable callus (Figs. 1A-C) were subsequently transferred to WPM containing cytokines either Kn or BAP alone (0, 0.1, 0.5, 1.0 and 2.0 mg/l). Interestingly, solely the friable callus exhibited diverse stages of somatic embryos (~10 somatic embryos per explant) within 8 weeks of transfer (Figs. 1D-F). Somatic embryos production was varied significantly with respect to origin of callus. Although all concentrations induced somatic embryos, Kn (0.5 mg/l) was found to be significantly better, which induced as high as 78% of somatic embryos from leaf derived calli followed by nodal segment (42%) (Table 1). While Kn was proved to be effective for induction of somatic embryo alone in *Begonia gracilis* (Castillo and Smith 1997) or in combination with 2, 4-D, were found to be more efficient in many other medicinal plants (Mahajan *et al.* 2016, Lee *et al.* 2020). BAP is also reported to be effective cytokinin for a number of other plant species such as *Piper hispidinervum* (de Sousa *et al.* 2022). Therefore, the variations in cytokine behavior for somatic embryo induction observed in this study are currently unclear but are believed to be due to species differences.

Further, to achieve synchronous embryogenesis, somatic embryos mass induced on WPM with Kn (0.5 mg/l) were incubated with ABA (0.1, 0.5 and 1.0 mg/l) on WPM solidified medium for 2 weeks (Table 2) and then subcultured on PGR free WPM for another 6 weeks. Amidst the combinations, it has been found that a maximum of 92% explant of leaf origin produced uniform somatic embryo, which became mature in Kn (0.5 mg/l) containing medium. Although all tested combinations produced mature somatic embryos regardless of the explant sources, ABA (1 mg/l) demonstrated a significantly higher conversion rate across all the explant sources used. Similarly, culturing on ABA favored the production of somatic embryos in different plant species (Walther *et al.* 2022, Chen *et al.* 2021). Frequency of somatic embryo maturation differed considerably on the basis of hormonal compositions and explant source. Among various plant growth regulators, ABA is commonly utilized for the maturation and synchronization of somatic embryos in medicinal plants (Kaur and Kapoor 2016) particularly because it inhibits precocious germination by conferring desiccation tolerance (Zhang *et al.* 2020, Sano and Poll 2021, Yan and Dong 2023) and promoting accumulation of storage lipids (Brookbank *et al.* 2021) and proteins (Zhou *et al.* 2023).

The transformation of somatic embryos into mature plants represents a bottleneck in the somatic embryogenesis procedure, sometimes due to the insufficient maturity of somatic embryos (Bantawa 2017). In this investigation, somatic embryos either failed to germinate or exhibited very low germination rates in a plant growth regulator (PGR)-free medium. These embryos at the cotyledonary stages (Fig. 1E) were isolated individually and placed onto medium supplemented with varying concentrations of Kn or BAP ranging from 0.1 to 2.0 mg/l, alongside GA_3 at 0.5 mg/l (Table 4). They showed germination within a four-week period (Figs. 1 G-H). Intriguingly, about 72.84% of somatic embryos derived from young leaf explants germinated when cultured on a medium containing 0.5 mg/l Kn and 0.5 mg/l GA_3 (Table

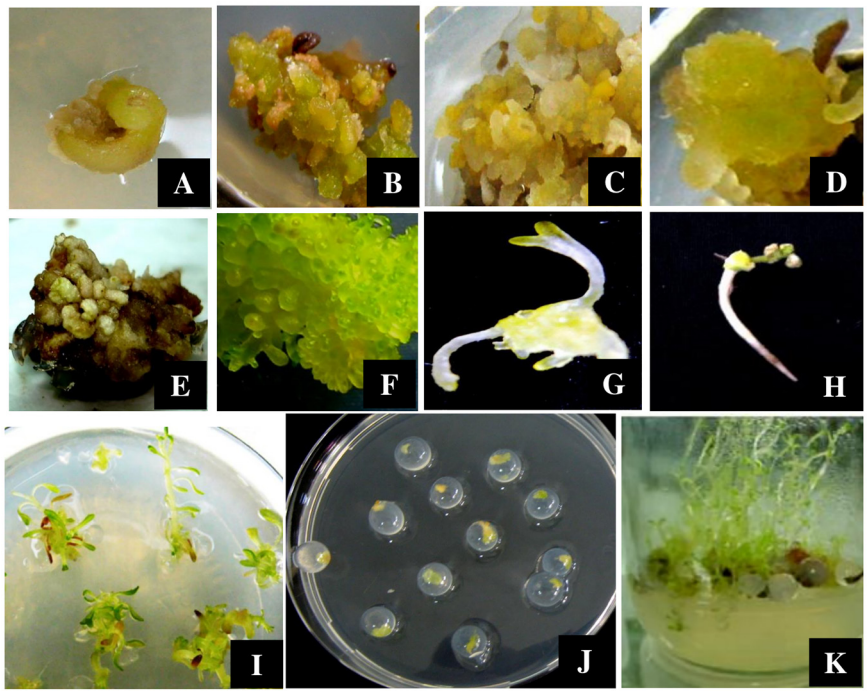

Fig. 1. Different stages of somatic embryos and synthetic seeds. (A) Initiation of callus from leaf explant. (B) Compact and non-friable callus. (C) Maturing firable callus. (D) Initiation of somatic embryos in leaf derived firable callus. (E) Cotyledonary stage of somatic embryo in WPM containing Kn (0.5 mg/l) + ABA (0.5 mg/l). (F) Greening of cotyledonary stage of somatic embryo. (G) Germinating somatic embryo in WPM supplemented with Kn (0.5) mg/l) + GA_3 (0.5 mg/l). (H) Germinating somatic embryo with distinct root and shoot. (I) Complete plantlet after germination of somatic embryos. (J) Germinating synthetic seeds at 0 day encapsulated in WPM basal medium. (K) Sprouting of encapsulated somatic embryos after 40 days of incubation.

3). This germination rate was significantly higher than that achieved with other tested combinations. Additionally, the source of explants was a crucial factor, with leaf-derived somatic embryos proving to be significantly superior to those from other explant sources. It is widely recognized that GA_3 is used by several workers for the initiation of somatic embryo germination in medicinal plants (e.g., Ilah *et al.* 2016, Igielski and Kępczyńska 2017). Distinct root and shoot were noted during germination of somatic embryos (Fig. 1H), which turned to complete plantlets within 3-4 weeks (Fig. 1I).

Synthetic seeds

The calcium alginate beads varied morphologically in texture, shape and transparency depending on the combinations of sodium alginate and $CaCl_2$ used. The optimal beads were formed with 3% sodium alginate and $CaCl_2.2H_2O$ (10.8 g/l) (Fig. 1 J). Using 1-2% sodium alginate resulted in unsuitable beads that were shapeless and too soft to handle, while 4-6% sodium alginate produced beads that were too hard, potentially causing significant delays in sprouting. Sprouting of encapsulated embryos of *P. scrophulariiflora* in controlled (0-day storage in WPM) started within the span of 12 days. Similar observation was made in *Citrus sinensis* (Mohamed *et al.* 2023). It's worth mentioning that all sprouts displayed normal root and shoot growth in this medium. On the other hand, WPM supplemented with Kn (0.5 mg/l) was found to be better than PGR free WPM (WPM0) as it produced higher multiple shoots which was morphologically normal.

Synthetic seeds sprouted only to those beads whose matrix was prepared using WPM nutrient. The alginate beads failed to sprout when the solution of $CaCl_2$ and sodium alginate were prepared using distilled water (Table 5). The lack of germination in

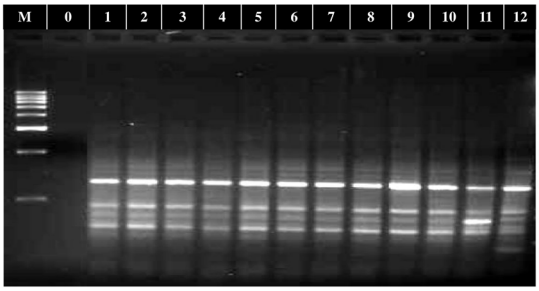

Fig. 2. The RAPD profile generated from the donor and somatic embryo-derived regenerated plantlets indicates genetic uniformity among them. M= Marker, 0= Negative control, 1 and 12= Donor plants and 2-11= Regenerated plantlets.

alginate beads prepared with distilled water could be attributed to inadequate nutrient availability. The germination rate of encapsulated somatic embryos decreased as the storage time increased. Many researchers (e.g., Mondal *et al.* 2013, Mohamed *et al.* 2023) have also observed a decline in germination when synthetic seeds were stored at lower temperatures for longer periods of time. A maximum of 100% survival was noted when stored up to 15 days, then after sprouting gradually declined to 87%, 74%, 70%, 58%, 42% and 20% when stored for 30, 45, 60, 75, 90 and 105 days respectively (Table 5). When the alginate beads prepared in WPM medium supplemented with Kn (0.5 mg/l) alone and subsequently stored for 0-45 days, germinating somatic embryos produced multiple shoots within 10-16 days (Fig. 1K). However, longer storage time at lower temperature led to the generation of a solitary shoot per bead. The time needed for germination also extended from 10-12 days (at 0 day of storage time) to 25 days (after 105 days of storage). There was no variation in sprouting frequency between 0-day and 15-day storage periods, both showing 100% germination.

Hardening

Fully *in vitro* grown plant derived from synthetic seed and encapsulated embryos were transferred from growing medium to plastic pots (6 cm × 8 cm) containing sterile forest black soil and sand (9:1) at the hot house of Kyoungnosla Nursery, Forest Department, Sikkim Government, India. After 8 weeks of time, 80% survival was noticed (data not shown) which were again transferred to larger polythene pots (8 cm × 10 cm) containing same potting mixture in nursery beds. Further, after 8 weeks of transfer, survival rate was finally found to be as high as 78% (data not shown).

Genetic fidelity

In our study, fingerprinting profiles of both donor and somatic embryo derived plants were generated by 40 random 10-mer primers. Monomorphic RAPD profile yielded a total of 151 alleles (Fig. 2), with an average of 3.75 fragments, ranging from 1 to 9 per primer, indicating homoginity among the micropropagated plants and genetic consistency identical to that of the donor (mother) plant. Characterization of *P. scrophulariiflora* at the genetic levels indicate that the protocol followed in our studies is capable of producing abundant plantlets derived from somatic embryos throughout the year, which is prerequisite for the conservation of the germplasm of this threatened medicinal plant. This study marks the initial generation of RAPD-based DNA fingerprinting profiles in plantlets derived from somatic embryos of *P. scrophulariiflora*, earlier our group (Bantawa *et al.* 2011a) also reported similar observation when plantlets of same species were directly regenerated from callus.

In conclusion, *Picrorhiza scrophulariiflora* stands out as a pharmaceutically significant endangered medicinal plant in the Eastern Himalayas. The population of this species has been declining gradually in the wild habitat of India (Bantawa *et al.* 2017, Bantawa 2017) due to over exploitation and unsustainable management. Therefore, this protocol may serve several purposes, (i) To multiply in large scale for conservation of such vulnerable species, (ii) To produce the secondary metabolite through bioreactor technology, (iii) For midterm preservation of somatic embryos (i.e. up to 105 days) of this species, (iv) For genetic manipulation of this species using leaf as explant.

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