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*Full Length Research Paper*

# Embryogenesis of *Gentiana straminea* and assessment of genetic stability of regenerated plants using inter simple sequence repeat (ISSR) marker

**Tao He[1,2]*, Lina Yang[1] and Zhigang Zhao[2]**

[1]Department of Biosciences, Qinghai University, Xining, Qinghai 810016, P. R. China.
[2]State Key Laboratory Breeding Base for Innovation and Utilization of Plateau Crop Germplasm, Qinghai University, Xining, Qinghai 810016, P. R. China.

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***Gentiana straminea*** **Maxim is an endangered medicinal plant in the Qinghai-Tibet Plateau. To speed up the production of this species, an *in vitro* protocol for efficient plant regeneration was developed from its leaf explants. Auxins and cytokinin alone or in combination were examined for their effects on callus induction and plant regeneration. The results indicated that Murashige and Skoog medium supplemented with 2 mg $l^{-1}$ 2,4-dichlorophenoxyacetic acid and 0.5 mg $l^{-1}$ N6-benzylaminopurine (BA) was the best medium for embryogenic callus induction, while 3 mg $l^{-1}$ BA induced the highest frequency (93.8%) of regeneration and development of shoots. Regenerated plantlets with well-developed root systems were hardened in the greenhouse and successfully established in the soil. Genetic stability of the regenerated plants was assessed by 25 inter simple sequence repeat (ISSR) markers. Out of 25 ISSR markers, 14 produced clear, reproducible bands with a mean of 6.9 bands per marker. The results confirmed that the regenerants maintained high genetic fidelity.**

**Key words:** *Gentiana straminea*, somatic embryogenesis, leaf explant, genetic stability, inter simple sequence repeat (ISSR).

## INTRODUCTION

*Gentiana straminea* Maxim is a perennial herb in the family Gentianaceae. It grows mainly in the alpine and subalpine belts in the Qinghai-Tibet Plateau at altitudes of 2500 to 4700 m (Guo, 1987). *G. straminea* are collected and used mostly for their medicinal effects. The roots of *G. straminea* contain the secondary metabolites gentiopicroside, swertiamarin, sweroside, qinjioside and macrophylloside, which possess unique medicinal properties against rheumatism, osteoarthritis, hepatitis, gastritis and cholecystitis (Yang, 1991). Gentianine has been produced from the plant and sold as capsules for the treatment of these diseases. Demand for the root stock of *G. straminea* in the pharmaceutical industry is met from the wild. As a result of over harvesting and lack of organized cultivation, *G. straminea* is now on the list of rare and threatened species in China. Therefore, it is imperative to develop appropriate tissue culture techniques for this species.

The tissue culture induced somaclonal variation which is quite common and can pose a problem to the genomic integrity of regenerated plants (Tyagi et al., 2010). Molecular techniques are at present powerful and valuable tools used in analysis of genetic fidelity of *in vitro* propagated plants. In comparison to amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) has been used most frequently,

*Corresponding author. E-mail: hetaoxn@yahoo.cn. Tel: +86-971-5310086.

**Abbreviations:** **AFLP,** Amplified fragment length polymorphism; **BA,** N6-benzylaminopurine; **CTAB**, cetyltrimethylammonium bromide; **dNTPs,** deoxyribonucleotide triphosphate; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **ISSR,** inter simple sequence repeat; **MS,** Murashige and Skoog; **NAA,** α-naphthaleneacetic acid; **PCR,** polymarase chain reaction; **RAPD,** randomly amplified polymorphic DNA; **RFLP,** restriction fragment length polymorphism; **UBC,** University of British Columbia.

because of its reproducibility, simplicity and cost-effectiveness (Liu et al., 2011). ISSR markers have been used successfully to assess genetic stability among *in vitro* regenerated plants of many plant species (Joshi and Dhwan, 2007; Chandrika et al., 2010; Zhang et al., 2010; Liu et al., 2011).

Recently, some species of *Gentianaceae* including *Gentiana cruciata* (Mikula et al., 2005), *Swertia chirayita* (Dhawan and Joshi, 2007), *Swertia chirata* (Chaudhuri et al., 2008), *Gentiana kurroo*, *Gentiana tibetica*, *Gentiana lutea*, *Gentiana pannonica* (Fiuk and Rybczynski, 2008) and *Gentiana triflora* (Doi et al., 2010) were studied for their regeneration. Plant regeneration of *G. straminea* has also been achieved via somatic embryogenesis from immature embryos (Cai et al., 2009). However, it is usually difficult to obtain immature embryos throughout the year, and the suitable stage for their culture is also strictly limited. The leaf is expected to be the most convenient explant since it has a high morphogenic potential, is easy to collect, can produce green mesophyll protoplasts, and is a potential target for transformation. In this study, we described another regeneration system based on initiation of embryogenic calli from leaf explants of *G. straminea*, and reported the assessment of clonal fidelity in the regenerated plants using ISSR markers. This protocol system is potentially simpler than regeneration from immature embryos.

## MATERIALS AND METHODS

Mature seeds were collected during the months of September and October from healthy plants of *G. straminea* growing in the northeastern Qinghai-Tibet Plateau in China. They were surface sterilized with 70% (v/v) ethanol for 45 s, then with 0.1% mercuric chloride ($Hgcl_2$; w/v) for 12 min, and rinsed five times with sterile distilled water. The seeds were germinated on half-strength Murashige and Skoog (MS) (1962) medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar without growth regulators. Leaves excised from 3-month-old seedlings were used for the explants.

### Induction of somatic embryogenesis

Leaf explants were cultured on MS medium supplemented with 1 to 4 mg $l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with N6-benzylaminopurine (BA) (0.5, 1 mg $l^{-1}$), 3% (w/v) sucrose and 0.65 g $l^{-1}$ agar. Cultures were incubated at 20 ± 2°C during the day and 15 ± 2°C at night with a 16-h photoperiod. A light intensity of 30 µmol $m^{-2}$ $s^{-1}$ was provided by cool-white fluorescent lamps. Explants forming embryogenic calli were scored after 6 weeks of culture and the percentage response was calculated.

Embryogenic calli were subcultured on MS medium containing different concentrations of 2,4-D (0.5 to 2 mg $l^{-1}$) for proliferation. Callus was subcultured at 2 weeks intervals.

### Plantlet regeneration

After 4 weeks of subculture, the embryogenic calli were transferred to MS medium supplemented with 0 to 4 mg $l^{-1}$ BA alone or in combination with α-naphthalene acetic acid (NAA) (0.5, 1 mg $l^{-1}$), 3% (w/v) sucrose and 0.65 g $l^{-1}$ agar for plantlet regeneration. Cultures were incubated at 20 ± 2°C during the day and 15 ± 2°C at night with a 16-h photoperiod. The regeneration frequency was scored after 6 weeks of culture. The regenerated plantlets (1 to 2 cm in height) were initially maintained on half-strength MS medium without growth regulators for rooting.

Plantlets with well-developed roots were removed from the culture medium, washed gently under running tap water, and transferred to plastic pots containing soil, and vermiculite (1:1). Plants were covered with transparent polyethylene bags to maintain adequate moisture for 2 weeks and transferred to the greenhouse (20°C during the day and 15°C at night, 16-h day-length and at 60% relative humidity). After a week, the plastic covering was gradually removed and the plantlets were maintained in the greenhouse in plastic pots containing normal garden soil until they were transplanted to the nursery.

### DNA extraction and ISSR analysis

Ten regenerated plants, originally derived from a single mother plant, were randomly selected from the hardening stage to screen their genetic integrity. Total DNA was extracted from fresh young leaves of regenerated plants and the mother plant using cetyl-trimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990) with minor modifications. Quantity of DNA was inspected by both gel electrophoresis and spectrometric assays.

A total of 25 ISSR primers were screened initially and 14 primers were selected in the study. Each reaction was performed in a total volume of 25 µl containing 2.5 µl of 10×PCR buffer (100 mM Tris-HCl, 500 mM KCl and 15 mM $MgCl_2$, pH 8.3), 0.8 µM primer, 0.2 mM deoxyribonucleotide triphosphate (dNTPs), 1.25 unit (U) of *Taq* DNA polymerase (TaKaRa, Japan,) and 25 ng genomic DNA. Amplifications were performed in a Thermal Cycler (Bio-Rad, U.S.A.). The reaction mixtures were denatured at 94°C for 5 min, followed by 36 cycles for 45 s at 94°C, 45 s at 55°C, 2 min at 72°C, and a final extension step of 7 min at 72°C and eventually stored at 4°C. Amplified products were analyzed by electrophoresis on 1.5% (m/v) agarose gel (TaKaRa, Japan) using 1×TBE buffer (Tris-Borate-EDTA buffer) at 5 V/cm for 100 min. The gels were stained with ethidum bromide (EB) solution. The amplified products were visualized and photographed under a UV transilluminator (LiuYi, China). A DL5000 DNA marker (TaKaRa, Japan) was used as the molecular weight standard.

### Statistical analysis

Each treatment consisted of 20 replicates and each experiment was repeated at least three times. Data were analyzed statistically using Duncan's multiple range test (Harter, 1960). For genetic analysis, only clear and reproducible bands were scored. Data were scored as 1 for the presence and 0 for the absence of a DNA band in each sample. The similarity coefficients were determined using the NTSYSpc 2.10s software package.

## RESULTS AND DISCUSSION

### Induction of embryogenic callus

Approximately 45% of *G. straminea* seeds germinated within two weeks of inoculation on the growth regulator-free MS medium. The seeds developed into plantlets (3 to 4 cm) consisting of five to six leaves within three

months of germination. Leaf explants from these seedlings were subsequently used for all the experiments.

Swelling and expansion in the explants were observed two to three days after culture initiation. Callus formation started after seven days from the cut ends of the leaf segments. After 4 weeks of culture, two types of callus were recognized. One type consisted of soft, watery, white non-embryogenic calli; the other consisted of compact, friable, yellow-green embryogenic calli (Figure 1A). Table 1 shows the effects of different concentrations of 2,4-D and BA on embryogenic callus formation. The induction frequency of embryogenic callus ranged from 48.4 to 82.8% depending on the 2,4-D concentrations. With an increase in the 2,4-D concentrations, the induction frequency was increased, but it declined at the highest concentration (4 mg $l^{-1}$). In general, auxins play a critical role in the induction of embryogenic callus. However, high concentrations of auxin restrain their further development. Choi et al. (2001) assumed that higher concentrations of 2,4-D might result in a greater possibility of somatic mutation. The use of cytokinins in combination with auxins has been reported to promote somatic embryogenesis induction (Liu et al., 2008; Capuana et al., 2007). Similarly, we observed that the addition of 0.5 mg $l^{-1}$ BA to the medium with 2,4-D (1 to 2 mg $l^{-1}$) resulted in an increase in embryogenic callus. Among the tests, 2 mg $l^{-1}$ 2,4-D and 0.5 mg $l^{-1}$ BA was the most effective, with the highest frequency (82.8%) of forming embryogenic callus (Table 1). In this study, either 2,4-D or a combination of 2,4-D with BA were effective in inducing embryogenic callus.

A previous study reported that leaves of *G. straminea* could be used to induce callus, but the frequency of callus formation was only 28.3% on the MB medium, they grew slowly and did not show embryogenesis (Cai et al., 2009). In our study, however, high frequency embryogenic calli from young leaves were achieved on the MS medium. This disparity may be due in part to the culture conditions. This study was conducted on the Qinghai-Tibet Plateau, which was the original region of *G. straminea*, but they were introduced into low altitude regions in the study of Cai et al. (2009). On the Qinghai-Tibet Plateau, the air was thin with an annual average barometric pressure of 684.2 h Pa (Li and Zhou, 1998). Carman (1988) found that the presence of low concentration of $O_2$ in the cultures might promote the formation of embryogenic callus in *Triticum aestivum*. This result was also observed in *Cydonia oblonga* (Fisichella and Morini, 2003). We assumed that the formation of embryogenic callus in our experiment was related to low $O_2$ in the gaseous environment. Furthermore, cultures were maintained at 20°C during the day and 15°C at night in our study, which approached the environment temperature of *G. straminea* growth. Similar culture condition was reported previously in *Saussurea laniceps*; an alpine plant growing in the Qinghai-Tibet Plateau (Chen and Li, 2005). It is useful for plant tissue culture to consider the environmental conditions in the original regions of the plant species. Further experiments to determine the effects of temperature and concentration of $O_2$ in the original regions of *G. straminea* on its *in vitro* culture are in progress.

Embryogenic callus clumps were transferred to the developing medium: MS medium containing different concentrations of 2,4-D (0.5 -2 mg $l^{-1}$) for subculture. On this medium, most of the calli became more compact and friable. Numerous proembryos appeared on the surface of calli within 2 weeks (Figure 1B). Previous research indicated that initiation of calli at a higher concentration and subsequent maintenance of calli at a low level of 2,4-D resulted in better development of embryogenic calli (Sahrawat and Chand, 2004). It was also found that the presence of a low concentration of 2,4-D in the medium was critical for embryogenic callus development in *G. straminea*. Here, 0.5 mg $l^{-1}$ 2,4-D may be the optimal concentration. These compact and organized callus tissues maintained their embryogenic potential for more than one year when the subculture in the medium was supplemented with 0.5 mg $l^{-1}$ 2,4-D. A cell suspension culture system for *G. straminea* was established using these calli.

**Plant regeneration**

The proembryos grew and developed to maturity when they were transferred to the regeneration medium, hormone-free MS medium or supplemented with BA (Table 2). Organized somatic embryos on the surface of calli were evident within two weeks and plantlet regeneration occurred in 21 days (Figure 1C to F). Usually, somatic embryos may germinate directly into complete plantlets on regeneration medium free of plant growth regulator (Cai et al., 2009; Vikrant and Rashid, 2003). Similar culture responses were also observed in our study. The regeneration frequency was 28.5% on the hormone-free medium (Table 2). However, we noted that the addition of BA to the regeneration medium was more efficient than the regeneration medium without growth regulator for plant regeneration from mature embryos, which was also reported previously (Cai et al., 2009; Liu et al., 2008). With an increase in the BA concentration, the regeneration frequency was increased, but it declined when the BA concentration increased to 4 mg $l^{-1}$. The highest regeneration frequency (93.8%) and average number of plantlets (31.7) per 1 g of callus were obtained on the MS medium containing 3 mg $l^{-1}$ BA (Table 2). Our results indicated that somatic embryogenesis of *G. straminea* required a high concentration of 2,4-D, but further maturation and germination of the embryos required the presence of BA.

The presence of NAA to the regeneration medium with BA significantly reduced the regeneration frequency as compared to the medium with BA alone. This effect was

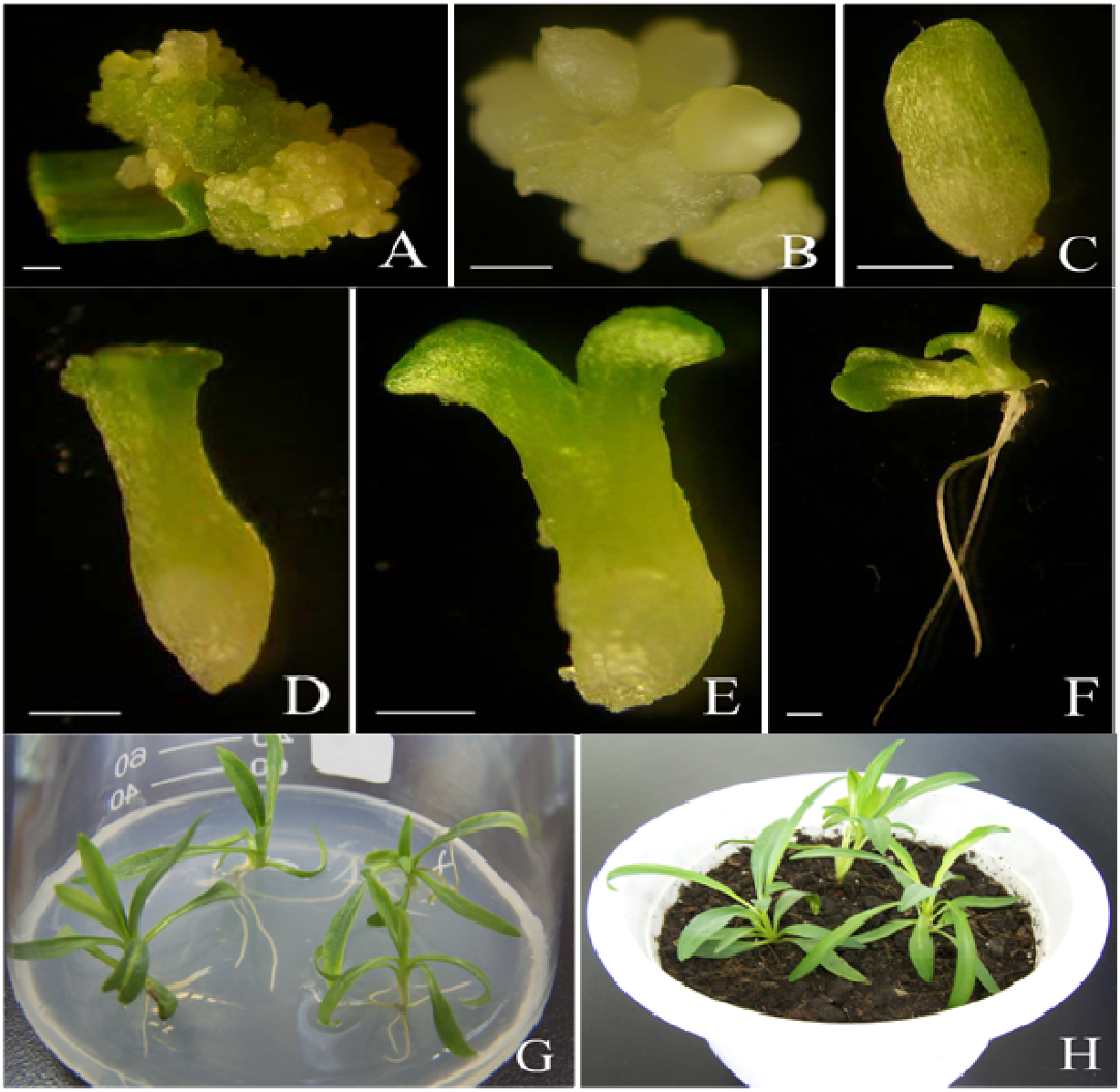


**Figure 1.** Somatic embryogenesis and plant regeneration in *G. straminea*. (A) Embryogenic calli from leaf explant; (B) proembryos on the surface of embryogenic calli; (C to E) somatic embryos at different development stages; (F) root and shoot development from somatic embryos; (G) plantlets regenerated through somatic embryogenesis; (H) acclimatized plantlets. Bars: 1.5 mm.

directly correlated to the NAA concentration, such that at the highest level tested (1.0 mg $l^{-1}$ NAA), plant regeneration was suppressed to a rate of 27.3% (Table 2). This was probably due to the fact that a few calli turned white, watery and died in the medium containing NAA and BA, whereas it did not occur in the medium with BA alone.

When green plantlets with or without primary roots were transferred to the half-strength MS medium without growth regulators, thick white roots developed in about four weeks (Figure 1G). Plantlets with well-developed roots were transferred to pots containing soil and vermiculite (1:1) and kept in a mist house for two weeks. After development of new leaves, plants were moved to a greenhouse. About 90% of the regenerated plants survived and exhibited normal growth (Figure 1H).

## ISSR analysis

True to type, clonal fidelity is one of the most important prerequisites in the tissue culture of any plant species (Chandrika et al., 2010). A major problem encountered with the *in vitro* culture is the presence of somaclonal

**Table 1.** Effect of 2,4-D and BA on embryogenic callus induction of leaf explants of *G. straminea*.

| 2,4-D (mg $l^{-1}$) | BA (mg $l^{-1}$) | Frequency of embryogenic callus induction (%) |
|---|---|---|
| 1 | 0 | 48.4±1.8$^{e}$ |
| 1 | 0.5 | 54.5±4.2$^{de}$ |
| 1 | 1 | 48.6±2.4$^{e}$ |
| 2 | 0 | 80.0±1.6$^{ab}$ |
| 2 | 0.5 | 82.8±3.8$^{a}$ |
| 2 | 1 | 75.0±3.9$^{bc}$ |
| 3 | 0 | 81.6±2.5$^{ab}$ |
| 3 | 0.5 | 76.2±1.2$^{bc}$ |
| 3 | 1 | 58.6±3.5$^{d}$ |
| 4 | 0 | 71.3±1.1$^{c}$ |
| 4 | 0.5 | 56.7±2.3$^{d}$ |
| 4 | 1 | 60.4±2.7$^{d}$ |

Evaluation was done six weeks after culture initiation. Values are means ± S.E. (standard error). Frequency of callus induction was calculated as (number of embryogenic calli / number of explants cultured) × 100. $^{a-e}$ Values followed by different letters in a column were significantly different at $P<0.05$ according to Duncan's multiple range test.

**Table 2.** Effect of BA and NAA on shoot regeneration of embryogenic calli of *G. straminea*.

| BA (mg $l^{-1}$) | NAA (mg $l^{-1}$) | Regeneration capacity of calli (%) | Number of shoot buds formed per 1 g of callus |
|---|---|---|---|
| 0 | 0 | 28.5±2.5$^{f}$ | 2.1±1.3$^{ef}$ |
| 1 | 0 | 80.3±1.0$^{bc}$ | 16.6±1.5$^{bc}$ |
| 1 | 0.5 | 75.0±3.1$^{c}$ | 7.3±2.1$^{de}$ |
| 1 | 1 | 63.6±3.1$^{d}$ | 4.8±1.8$^{ef}$ |
| 2 | 0 | 85.7±2.3$^{b}$ | 20.3±2.7$^{b}$ |
| 2 | 0.5 | 33.3±2.7$^{ef}$ | 2.4±1.1$^{ef}$ |
| 2 | 1 | 57.1±2.9$^{d}$ | 8.1±1.7$^{de}$ |
| 3 | 0 | 93.8±1.7$^{a}$ | 31.7±4.6$^{a}$ |
| 3 | 0.5 | 37.3±1.9$^{e}$ | 1.6±0.9$^{f}$ |
| 3 | 1 | 27.3±2.9$^{f}$ | 0.7±0.5$^{f}$ |
| 4 | 0 | 81.7±1.3$^{b}$ | 11.3±1.5$^{cd}$ |

Evaluation was done six weeks after transfer of embryogenic calli to regeneration medium. Values are means ± S.E. (standard error). Regeneration capacity of calli was calculated as (number of calli with green shoots / number of calli plated on regeneration medium) × 100. $^{a-f}$, Values followed by different letters in a column were significantly different at $P<0.05$ according to Duncan's multiple range test.

variation. In this study, ISSR profiles were used to check genomic variation. To select suitable primers for the study of *G. straminea*, 25 ISSR primers were screened using a mother plant DNA sample. Out of these 25 primers, only 14 produced more than four clear and scorable bands, and were used in further PCR analysis. 96 scorable bands were selected, ranging in size from 200 bp to 3 kb (Table 3). The number of bands per each primer varied from 4 to 13, with an average of 6.9 bands per primer. A total of 1023 bands were produced by ISSR marker, giving rise to monomorphic patterns across 11 plantlets analyzed. The similarity coefficient among the plants ranged from 0.93 to 0.99 with a mean of 0.96. Ten regenerants did not deviate at all from the parental genotype. A sample of monomorphic gels obtained from ISSR primers (UBC817) is shown in Figure 2. The ISSR analysis of *G. straminea* revealed a low variation among regenerants which is similar to *Anoectochilus formosanus* (Zhang et al., 2010), *Nothapodytes foetida* (Chandrika et al., 2010) and *Hydrangea macrophylla* (Liu et al., 2011) *in vitro* regenerated plants. Somatic embryogenesis has been considered as an effective pathway of plant

**Table 3.** List of ISSR primers used to screen the genetic stability of regenerated plantlets of *G. straminea*.

| UBC Primers | Sequence (5′-3′) | Number of scorable bands per primer | Size range (bp) |
|---|---|---|---|
| 807 | AGAGAGAGAGAGAGAGT | 8 | 250-2000 |
| 810 | GAGAGAGAGAGAGAGAT | 10 | 400-2500 |
| 811 | GAGAGAGAGAGAGAGAC | 4 | 250-1000 |
| 812 | GAGAGAGAGAGAGAGAA | 4 | 400-1500 |
| 817 | CACACACACACACACAA | 9 | 200-1500 |
| 825 | ACACACACACACACACT | 5 | 300-1500 |
| 826 | ACACACACACACACACC | 4 | 500-1500 |
| 827 | ACACACACACACACACG | 9 | 250-1500 |
| 836 | AGAGAGAGAGAGAGAGYA | 13 | 250-3000 |
| 857 | ACACACACACACACACYG | 9 | 300-2000 |
| 859 | TGTGTGTGTGTGTGTGRC | 4 | 250-2000 |
| 866 | CTCCTCCTCCTCCTCCTC | 5 | 300-2000 |
| 873 | GACAGACAGACAGACA | 7 | 300-2000 |
| 890 | VHVGTGTGTGTGTGTGT | 5 | 200-750 |

UBC: University of British Columbia. Y = (C, T), R = (A, G), V = (A, C, G), H = (A, C, T).

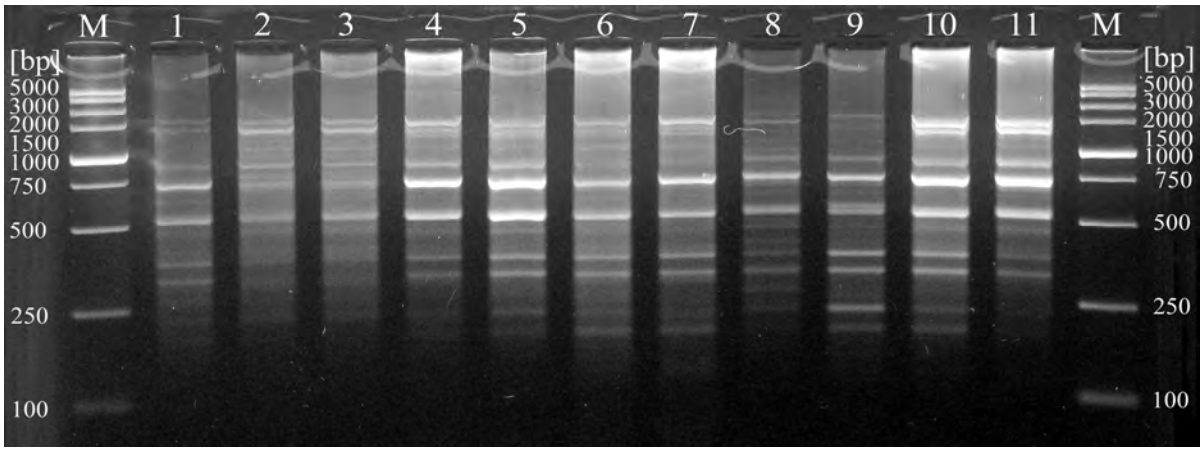


**Figure 2.** ISSR products generated from a mother plant (1), and regenerated plantlets (2 to 11) of *G. straminea* using primer UBC 817. M: DL5000.

regeneration because of its high regenerative potential and lower risk of chimeric mutations (Chen and Chang, 2000). The results obtained demonstrated that *G. straminea*, regenerated through somatic embryogenesis, maintained high genetic fidelity.

In conclusion, an efficient protocol was established for somatic embryogenesis and plant regeneration from leaf explants of an endangered medicinal plant, *G. straminea*. This method is a reliable mode for maintaining genetic stability. This *in vitro* technique should be a very useful tool for the conservation and genetic engineering of *G. straminea*.

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