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Full Length Research Paper

Micropropagation of *Anisodus tanguticus* and assessment of genetic stability of regenerated plants using inter simple sequence repeat (ISSR) marker

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For the first time, an *in vitro* protocol for efficient shoot multiplication has been developed from shoot tip explants of *Anisodus tanguticus*, an endangered medicinal plant in the Qinghai-Tibet Plateau. Multiple shoots were induced from the shoot tip explants on Murashige and Skoog (MS) medium supplemented with various concentrations of N6-benzylaminopurine (BA), kinetin (KT) or in combination with α-naphthalene acetic acid (NAA). The presence of BA was more effective than KT on shoot multiplication. A maximum of 3.2 shoots were obtained per explant on MS medium supplemented with 2 mg L^{-1} BA. Addition of 0.5 or 1 mg L^{-1} NAA to the BA-containing medium promoted callus formation and reduced shoot multiplication. Shoots treated with 0.5 mg L^{-1} indole-3-acetic acid (IAA) showed the highest average root number (4.08) and the highest percentage of rooting (75.2%). Micropropagated plantlets 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, 18 markers produced clear, reproducible bands with a mean of 6.7 bands per marker. The results confirmed that the regenerants maintained high genetic fidelity. This *in vitro* technique may help in the conservation and propagation of *Anisodus tanguticus*.

Key words: *Anisodus tanguticus*, micropropagation, shoot multiplication, genetic stability, inter simple sequence repeat (ISSR).

INTRODUCTION

Anisodus tanguticus (Maxim.) Pascher is a perennial herb in the family Solanaceae. It grows mainly in the alpine and subalpine belts in the Qinghai-Tibet Plateau at altitudes from 2,200 to 4,200 m (Guo, 1987). *A. tanguticus* are collected and used mostly for their medicinal effects. The roots of *A. tanguticus* contain the secondary metabolites anisodine, anisodamine, scopolamine and atropine, which possess anaesthetic and tranquil properties (Yang, 1991). It has a significant impact in China as one of the 50 traditional herbs used in traditional Chinese medicine. Demand for the root stock of *A. tanguticus* in the pharmaceutical industry is met from the wild. As a result of over harvesting and lack of organized cultivation, *A. tanguticus* is now on the list of rare and threatened species in China. *A. tanguticus* regenerates through roots and seeds. However, its germination is low and progresses slowly under natural conditions (He and Jia, 2009). Therefore, it is imperative to develop appropriate tissue culture techniques for this species.

The tissue culture induced somaclonal variation and is quite common and can pose a problem to the genomic

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Abbreviations: **AFLP,** Amplified fragment length polymorphism; **BA,** N6-benzylaminopurine; **CTAB,** cetyltrimethylammonium bromide; **dNTPs,** deoxyribonucleotide triphosphate; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **ISSR,** inter simple sequence repeat; **KT,** kinetin; **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.

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, 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 (He et al., 2011; Liu et al., 2011).

The present investigation describes an efficient protocol for regeneration of shoots from shoot tip explants of *A. tanguticus*, and reports the assessment of clonal fidelity in the regenerated plants using ISSR markers. To our knowledge, this is the first report of tissue culture of the species and the analysis of DNA sequence variation in micropropagated plantlets of *A. tanguticus*.

MATERIALS AND METHODS

Plant material

Mature seeds were collected during the months of September and October from healthy plants of *A. tanguticus* 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 seed dormancy was broken using the method described previously (He and Jia, 2009). Seeds were germinated on half-strength Murashige and Skoog (1962) (MS) medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar without growth regulators. Shoot tips (0.5 to 1 cm in length) excised from 8 weeks-old seedlings were used for the explants.

Culture media and conditions

Shoot tip explants were cultured on MS medium supplemented with 1, 2, or 3 mg L^{-1} N6-benzylaminopurine (BA), kinetin (KT) or in combination with 0.5, or 1 mg L^{-1}α-naphthalene acetic acid (NAA) for shoot multiplication. Explants grown on MS medium without plant growth regulators were used as control. All media were supplemented with 3% (w/v) sucrose and 0.65% (w/v) agar.

The pH of the media was adjusted to 5.8 with 1 N NaOH before adding agar. Media were autoclaved at 1.05 kPa at 121°C for 20 min. Cultures were maintained at 25±2°C under a 16 h photoperiod at a photosynthetic flux of 30 µmol m^{-2} s^{-1}, provided by cool daylight fluorescent lamps. After 6 weeks of culture, explants were evaluated in terms of frequency, length, and number of shoots per explant.

Rooting

The shoots (2 to 3 cm in length) were cultured on half-strength MS medium supplemented with 0, 0.25, or 0.5 mg L^{-1} NAA, indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) for rooting, respectively. Cultures were maintained at 25±2°C under a 16 h photoperiod at a photosynthetic flux of 30 µmol m^{-2} s^{-1}, provided by cool daylight fluorescent lamps. All media were supplemented with 3% (w/v) sucrose and 0.65% (w/v) agar. Rooting response was evaluated after 6 weeks of culture.

Plant acclimatization

Plantlets with well-developed roots were removed from the culture medium, washed gently under running tap water, and transferred to plastic pots containing soil, vermiculite (1:1). Plants were covered with transparent polyethylene bags to maintain adequate moisture for 2 weeks and transferred to the greenhouse. 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 micropropagated plants and the mother plant using cetyltrimethylammonium 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 inter simple sequence repeat (ISSR) primers were screened initially and 18 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

Establishment of aseptic plantlets

Approximately seventy-two percent of *A. tanguticus* seeds germinated within 2 weeks of inoculation on a growth regulator-free MS medium. The seeds developed into plantlets (3 to 4 cm) consisting of 5 to 6 leaves within 8 weeks of germination. Shoot tip explants from these

Table 1. Effect of cytokinins and auxin on induction of multiple shoots from shoot tip explants of *Anisodus tanguticus* on MS medium after 6 weeks of culture.

BA (mg l^{-1})	KT(mg l^{-1})	NAA (mg l^{-1})	Percent response[1]	Number of shoots per explant	Average shoot length (cm)
0	0	-	0	0[g2]	0[e]
0.5	-	-	89.4	2.67±1.53[b]	0.54±0.22[a]
1	-	-	87.5	2.50±1.03[bc]	0.40±0.15[bc]
2	-	-	94.6	3.20±1.23[a]	0.56±0.29[a]
3	-	-	66.7	1.78±0.65[ef]	0.33±0.12[c]
-	0.5	-	61.8	2.01±1.22[de]	0.46±0.19[ab]
-	1	-	76.9	2.09±1.13[d]	0.39±0.17[bc]
-	2	-	90.1	2.63±0.55[b]	0.60±0.37[a]
-	3	-	81.2	2.21±0.45[cd]	0.36±0.12[bc]
1	-	0.5	78.7+	1.96±0.68[de]	0.53±0.38[a]
2	-	0.5	80.5+	2.12±1.16[d]	0.48±0.15[ab]
3	-	0.5	57.1+	1.59±0.89[f]	0.30±0.09[cd]
1	-	1	75.3+	1.75±0.96[ef]	0.51±0.36[a]
2	-	1	77.2+	1.52±0.87[f]	0.35±0.17[c]
3	-	1	62.5+	1.92±0.93[de]	0.22±0.12[cd]

[1]+ Callus formed at the shoot base. [2]Values represent mean ± Standard error. Values followed by the same *letter* in each *column* are not significantly different ($p \leq 0.05$).

plantlets were subsequently used for all experiments.

Shoot proliferation and multiplication

Shoot proliferation was initiated within 7 to 8 days on the MS medium containing different cytokinins. However, explants cultured in the growth regulator-free media were not responsive, as no adventitious shoots were observed in the media (Table 1). The effect of BA and KT on shoot regeneration frequency, shoot number per explant was differential. The presence of BA promoted a higher rate of shoot multiplication than KT. These results were different from the observations of *Pinus kesiya*, where KT and BA were equally effective for shoot formation (Nandwani et al., 2001). This disparity may be due in part to the different organs or species used. The promotive role of BA for shoot differentiation has been documented in *Searsia dentate* (Prakash and Staden, 2008) and *Holarrhena antidysenterica* (Kumar et al., 2005). With an increase in the BA concentration, the percent response and the number of shoots per explants were increased. However, they declined when the BA concentration increased to 3.0 mg L^{-1} (Table 1). Similar culture responses were also observed on medium containing KT. It was also found that the shoot length was reduced with an increase in the BA or KT concentration, which was also reported previously (Haw and Keng, 2003; Gyana, 2004). The highest shoot regeneration frequency (94.6%) and number of regenerated shoots (3.2) were observed on the MS medium containing 2 mg L^{-1} BA (Table 1 and Figure 1A).

Addition of NAA to BA-containing media accentuated the morphogenic response and facilitated the growth of shoots (Prakash and Staden, 2008). Our results did not exactly agree with that report, but we did find that the addition of NAA to the medium promoted callus formation at the shoot base (Table 1 and Figure 1B). These results were also observed in *Scabiosa caucasica* (Hosoki and Nojima, 2004). Marks and Simpson (1994) suggested that this callus formation might be due to the action of accumulated auxin at the basal cut ends, which stimulated cell proliferation. Addition of NAA with increasing concentrations reduced the number of regenerated shoots per explant (Table 1).

Rooting

Elongated shoots (2 to 3 cm) were excised from cultures, and transferred to half-strength MS medium supplemented with different auxins for rooting. Rooting initiation was observed within 7 to 14 days in all treatments. The highest frequency of root formation were achieved on medium with 0.5 mg L^{-1} IAA (75.2%), followed by IBA at 0.5 mg L^{-1} (51.4%). The addition of NAA either at 0.25 or 0.5 mg L^{-1} resulted in lower percentages of rooting as compared to those obtained with IAA and IBA, but significantly higher than control (12.5% rooting). Exogenous auxins are often used in a number of plant species to promote *in vitro* rooting of micro-shoots (Cao et al., 2007; Purkayastha et al., 2008). The presence of auxin greatly increased the rooting frequency (Table 2). The maximum root number (4.08) and root length (1.31 cm) were achieved on medium with 0.5 mg L^{-1} IAA (Figure 1C) and 0.25 mg L^{-1} IBA,

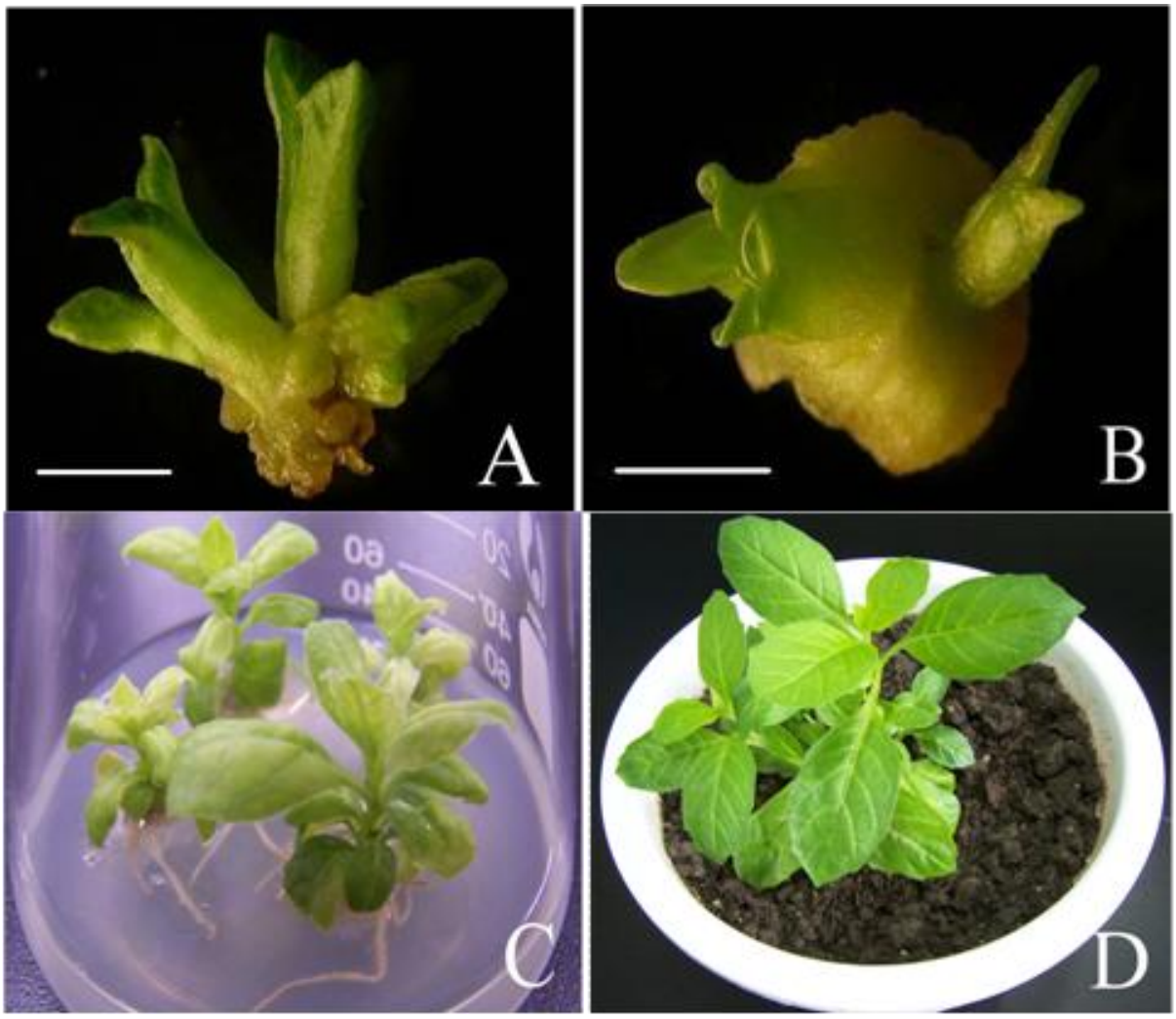

Figure 1. Micropropagation of *Anisodus tanguticus*. (A) Multiple shoot development from a shoot tip explant on MS medium supplemented with 2 mg L^{-1} BA. (B) Development of multiple shoots with callus from a shoot tip explant on MS medium supplemented with 2 mg L^{-1} BA and 0.5 mg L^{-1} NAA. (C) Root initiation from micro-shoots on half-strength MS medium supplemented with 0.5 mg L^{-1} IAA. (D) Rooted plants established in soil. Bars: 0.5 cm.

respectively. Root length with the absence of growth regulators (0.12 cm) was significantly inferior compared to the other treatments. Previous research has indicated that IAA and IBA were most effective in inducing roots (Iapichino and Airo, 2008). The present study also demonstrated that the superiority of IAA and IBA over NAA in root formation. Iapichino and Airo (2008) assumed that the negative effect of NAA was related to its longer persistence compared to other auxins, remaining in tissue in free form and blocking root emergence.

Acclimatization

The rooted plantlets were transferred to pots containing soil, vermiculite (1:1) and kept in a mist house for 2 weeks. After the development of new leaves, plants were moved to a greenhouse. About 90% of the micropropagated plants survived and exhibited normal growth (Figure 1D).

ISSR analysis

True to type clonal fidelity is one of the most important prerequisites in the micropropagation of any plant species (Chandrika et al., 2010). A major problem encountered with the *in vitro* culture is the presence of somaclonal variation. In this study, ISSR profiles were used to check genomic variation. To select suitable primers for the study of *A. tanguticus*, 25 ISSR primers

Table 2. Effect of auxins on rooting response of *in vitro* regenerated shoots of *Anisodus tanguticus* on half-strength MS medium after 6 weeks of culture.

NAA (mg L^{-1})	IAA (mg L^{-1})	IBA (mg L^{-1})	Percent response	Number of roots per shoot	Average root length (cm)
0	0	0	12.5	0.14±0.08[e1]	0.12±0.05[e]
0.25	-	-	20.1	0.20±0.15[e]	0.23±0.10[de]
05	-	-	32.3	0.67±0.21[d]	0.31±0.11[d]
-	0.25	-	34.5	0.75±0.50[d]	0.53±0.25[c]
-	0.5	-	75.2	4.08±2.37[a]	0.92±0.59[b]
-	-	0.25	33.7	2.15±1.47[b]	1.31±0.93[a]
-	-	0.5	51.4	1.53±0.91[c]	0.38±0.14[cd]

[1]Values represent mean ± Standard error. Values followed by the same *letter* in each *column* are not significantly different ($p \leq 0.05$).

Table 3. List of ISSR primers used to screen the genetic stability in micropropagated plantlets of *Anisodus tanguticus.*

UBC Primers	Sequence (5′-3′)	Number of scorable bands per primer	Size range (bp)
807	AGAGAGAGAGAGAGAGT	7	150-2000
810	GAGAGAGAGAGAGAGAT	8	300-3000
815	CTCTCTCTCTCTCTCTG	5	250-3000
817	CACACACACACACACAA	7	250-1500
824	TCTCTCTCTCTCTCTCG	5	500-3000
825	ACACACACACACACACT	10	250-1500
826	ACACACACACACACACC	6	300-1500
827	ACACACACACACACACG	5	300-1500
834	AGAGAGAGAGAGAGAGYT	7	150-2000
836	AGAGAGAGAGAGAGAGYA	8	300-2000
840	GAGAGAGAGAGAGAGAYT	4	500-2000
844	CTCTCTCTCTCTCTCTRC	5	400-2000
857	ACACACACACACACACYG	6	400-3000
859	TGTGTGTGTGTGTGTGRC	6	300-3000
864	ATGATGATGATGATGATG	7	250-3000
866	CTCCTCCTCCTCCTCCTC	5	250-1000
887	DVDTCTCTCTCTCTCTC	11	300-2000
890	VHVGTGTGTGTGTGTGT	8	300-2000

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

were screened using a mother plant DNA sample. Out of these 25 primers, only 18 primers produced more than four clear and scorable bands, and were used in further PCR analysis. 120 scorable bands were selected, ranging in size from 150 bp to 3 kb (Table 3). The number of bands per each primer varied from 4 to 11, with an average of 6.7 bands per primer. A total of 1284 bands were produced by ISSR marker, giving rise to monomorphic patterns across 11 plantlets analyzed. The similarity coefficient among the plants ranged from 0.95 to 0.98 with a mean of 0.97. Ten regenerants did not deviate at all from the parental genotype. A sample of monomorphic gels obtained from ISSR primers (UBC887) is shown in Figure 2. The ISSR analysis of *A. tanguticus* revealed a low variation among regenerants which is similar to *Nothapodytes foetida* (Chandrika et al., 2010), *Gentiana straminea* (He et al., 2011) and *Hydrangea macrophylla* (Liu et al., 2011) *in vitro* regenerated plants. The results obtained demonstrated that *A. tanguticus*, multiplied by shoot tips, maintained high genetic fidelity.

In conclusion, an efficient protocol was developed for successful micropropagation of an endangered medicinal plant, *A. tanguticus*. This method was a reliable mode for maintaining genetic stability. This *in vitro* technique may help in the conservation and propagation of the species and possibly lead to the synthesis and extraction of active compounds from root sources.

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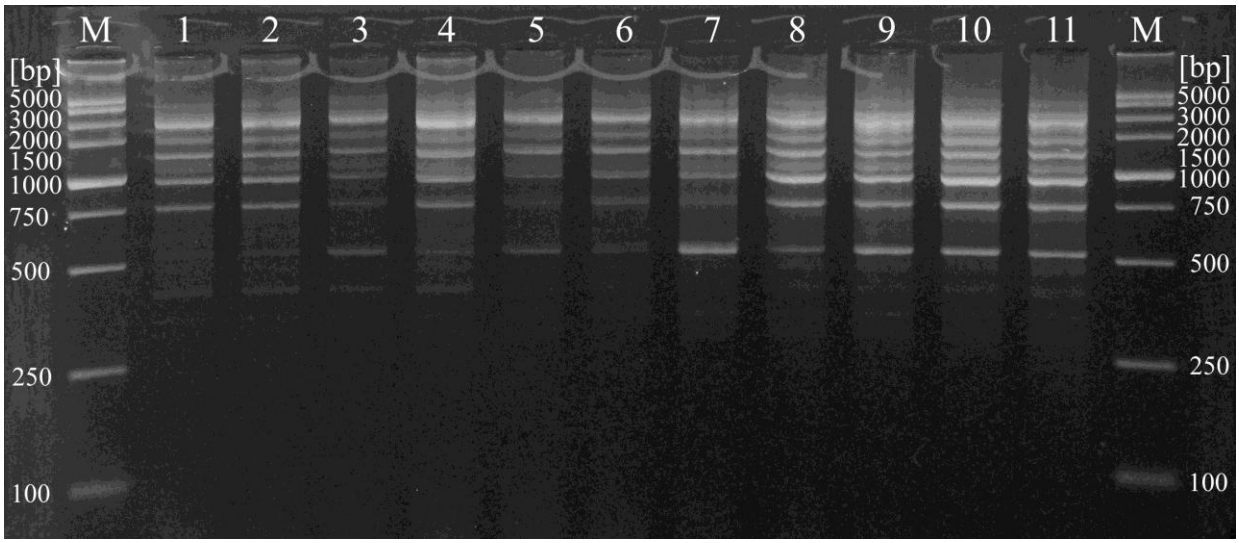

Figure 2. ISSR products generated from a mother plant (1) and micropropagtated plantlets (2 to 11) of *Anisodus tanguticus* using primer UBC 887. M: DL5000.

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