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Full Length Research Paper

High efficiency regeneration and genetic stability analysis of somatic clones of *Gynura bicolor* DC.

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***Gynura bicolor* DC. is a perennial vegetable and medicinal plant. It is an important source of anthocyanins. The effects of different growth regulators on callus induction and plant regeneration were evaluated. The best SFC index (8.6) of plant regeneration was obtained in combination of 2,4-D at 2.0 mg/l and BA at 0.5 mg/l, and the frequency of regenerating explants was 78.3%. The highest number of shoots per explant was 11. The genetic stability of the regenerants was analyzed by random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) molecular markers and flow cytometry. The results indicated that no somaclonal variation was detected among the regenerants. To our knowledge, this is the first report of somatic clone study in *G. bicolor*. The high efficient and reproducible protocol will be advanced for the further studies on secondary metabolic products, transformations and breeding of this potential medicinal plant.**

Key words: Flow cytometry analysis, genetic stability, *Gynura bicolor*, inter-simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), regeneration.

INTRODUCTION

Gynura bicolor DC. is a perennial vegetable belonging to Asteraceae and is originated from the tropical area of East Asia. *G. bicolor* contains relatively high levels of vitamin C, crude protein and essential amino acids. Moreover, *G. bicolor* is a rich source of anthocyanins which have important medicinal values (Yoshitama et al., 1994). Anthocyanins are believed to have important therapeutic values, including antitumor, antiulcer, antioxidant and anti-inflammatory activities (Wang et al., 1999; Schirrmacher et al., 2004). *G. bicolor* hot water extract and the SepPakC18 column adsorbed fraction showed inhibitory effect of the HL60 human leukemia cell growth (Hayashi et al., 2002).

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Abbreviations: BA, 6-Benzylaminopurine; **MS,** Murashige and Skoog (1962) medium; **NAA,** a-naphthalene acetic acid; **TDZ,** thidiazuron; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **KT,** kinetin; **IBA,** indole 3-butyric acid; **$AgNO_3$,** silver nitrate; **CTAB,** cetyl trimethylammonium bromide; **ISSR,** inter-simple sequence repeat; **RAPD,** random amplified polymorphic DNA; **FCM,** flow cytometry.

In vitro cell and tissue cultures are frequently used for the production of secondary metabolites (Zhao and Verpoorte, 2007; Dörnenburg, 2008), therefore, *in vitro* tissue culture of *G. bicolor* may be a potential method for the production of large amounts of anthocyanins for medicinal applications. The present regeneration protocol is useful for large-scale clonal multiplication as well as for transformation studies.

In plant regeneration, one of the most crucial concerns is to retain genetic integrity with respect to the mother plants. Some strategies are available for detecting genetic variations, including DNA analysis techniques and flow cytometry analysis (Fiuk et al., 2010). It is important to detect the variations of somatic clones at early stage during plant tissue culture to avoid further labors and supplies consumption (Qin et al., 2006, 2007). The randomly amplified polymorphic DNA (RAPD) (Williams et al., 1990) and the inter-simple sequence repeat (ISSR) (Zietjiewicz et al., 1994) have been successfully employed to assess the genomic stability in regenerated plants including those with no obvious phenotypic alterations (Tyagi et al., 2010; Samantaray and Maiti, 2010). Flow cytometry was performed to study DNA content stability of

Table 1. Effects of different combinations and concentrations of plant growth regulators on callus and adventitious shoot formation in *G. bicolor.*

Growth regulator (mg/l)	Callus formation (%)	Callus browning rate (%)	Frequency of regenerating explants (%)	Number of shoot per explant	SFC
2,4-D(1.0)+ BA(0.1)	100	21.7±2.9^{c}	76.7±2.9^{a}	10.3±1.0^{a}	7.9
2,4-D(1.0)+ BA(0.5)	100	20.0±5.0^{c}	73.3±7.6^{a}	10.5±1.1^{a}	7.7
2,4-D(2.0)+ BA(0.1)	100	18.3±2.9^{c}	73.3±5.8^{a}	10.7±1.2^{a}	7.8
2,4-D(2.0)+ BA(0.5)	100	16.7±2.9^{c}	78.3±2.9^{a}	11.0±0.9^{a}	8.6
2,4-D(2.0)+ KT(0.5)	100	3.3±2.9^{d}	13.3±5.8^{d}	2.0±0.9^{c}	0.3
NAA(2.0)+ BA(0.5)	100	66.7±5.8^{a}	38.3±2.9^{c}	9.8±0.8^{a}	3.8
NAA(2.0)+KT(0.5)	100	68.3±2.9^{a}	36.7±2.9^{c}	8.5±1.2^{b}	3.1
2,4-D(0.3)+ BA(3.0)	100	36.7±7.6^{b}	48.3±7.6^{b}	10.0±0.9^{a}	4.8
NAA(0.3)+BA(3.0)	100	68.3±7.6^{a}	43.3±2.9^{bc}	10.2±0.8^{a}	4.4

Each value represents the mean ± SE of three repeated experiments each with three replicates (20 explants each). Means followed by the same letters were not significantly different according to the LSD test at the 5 % level of probability. Data were scored after 15 weeks of culture initiation. SFC = average number of shoots per regenerating explant × (% regenerating explants) / 100.

regenerated plants which offers the possibility of fast and large scale analysis of the DNA content of cells for various purposes such as, determination of species specific DNA amount, analysis of the cell cycle activity in different tissues and measurement of endopolyploidization levels (Ćalić-Dragosavac et al., 2010; Guo et al., 2009).

This study described an efficient system for the plant regeneration of *G. bicolor.* The genetic variation for the regenerated plants was analyzed by RAPD, ISSR and flow cytometry techniques. High efficient protocol for producing tissue cultural based regenerants without somatic genetic variations would be useful for further studies on secondary metabolic products, gene transformations and plant breading of this potential medicinal plant species.

MATERIALS AND METHODS

Culture medium

The leaf explants were cultured on MS medium supplemented with different combinations of plant growth regulators. All the media were solidified with agar (8 g/l), adjusted to pH 5.8 by 0.1 N NaOH and sterilized by autoclaving at 121°C for 20 min. All plant growth regulators were incorporated in the medium before autoclaving. $AgNO_3$ was added to the medium after autoclaving by filter sterilization.

Culture conditions

G. bicolor genotype Hongye was used in this study. Tissues of young and fully expanded leaves were used as explants for the culture. Leaves were surface-sterilized in 70% ethanol for 30 s, followed by immersion in mercuric chloride (0.1 % w/v) with two drops of Tween-20 for 8 min. The plant materials were subsequently rinsed three times in sterile water.

The leaves were cut into 1 cm^2 pieces and were used as explants. Ten explants were inoculated in a 90 mm Petri dishes containing 25 cm^3 solidified medium. Leaf explants were placed with the adaxial surface towards the medium and then incubated in a growth chamber at 26 ± 2°C with a photoperiod of 14 h and light intensity of 40 µmol m^{-2} s^{-1} provided by fluorescent tubes. The same culture conditions were used in all the experiments.

Effect of plant growth regulators on callus and multiple shoot bud formation

Callus was induced on explants by culturing on a medium containing various levels of NAA, 2,4-D, KT and BA (Table 1); combinations of growth regulators were designed according to the previous results obtained from preliminary experiments). After four-week callus induction, the calli were transferred to shoot induction medium containing TDZ at 0.5 mg/l and $AgNO_3$ at 4 mg/l. The calli were subcultured every four weeks. The number of explants with shoot buds was scored after 15 weeks culture and the adventitious shoots formed per explant were counted.

The SFC index (shoot forming capacity; Lambardi et al., 1993) was calculated as follows:

SFC = average number of shoots per regenerating explant × (% regenerating explants) / 100 (1)

Rooting and plant establishment

When shoots reached 5 mm in length, the shoots were excised from the explants and placed on shoot elongation medium containing TDZ at 0.2 mg/l and $AgNO_3$ at 4 mg/l. The elongated shoots were transferred to rooting medium containing IBA at 0.5 mg/l. After rooting, the plantlets were transferred to pots containing peat and perlite (2:1) and placed in a greenhouse under plastic covering to maintain a high humidity.

DNA extraction and PCR amplification conditions

Forty (40) random-selected regenerated plants along with the mother plant were used for RAPD and ISSR analysis. Total genomic DNA was extracted from leaves of each individual using CTAB method (Guo et al., 2003). Totally, 36 arbitrary 10-mer RAPD primers were used for the RAPD analysis following the method of Williams et al. (1990). RAPD amplifications were performed routinely using PCR mixture (20 µl) which contained 25 ng of genomic DNA as template, 2.0 µl of 10× PCR buffer (1.5 mM $MgCl_2$), 200 µM dNTPs, 1 unit (U) of Taq DNA polymerase (Takara Shuzo Co.) and 1 µM of each prime (Table 2). The optimized PCR

Table 2. Detailed information on the primers used in the RAPD and ISSR analysis of genetic stability in regenerants and number of scorable bands for each primer.

RAPD Primer	Sequence (5'-3')	Tm (℃)	Number of scorable band	RAPD Primer	Sequence (5'-3')	Tm (℃)	Number of scorable band	ISSR Primer	Sequence (5'-3')	Tm (℃)	Number of scorable band
C-1	CAGAAGCGGA	36.9	0	C-19	ACCCGGTCAC	41	6	S1	$(AC)_8G$	58	4
C-2	TGAGCCTCAC	36.9	4	C-20	CTGCTGGGAC	41	5	S2	$(AC)_8C$	58	6
C-3	GGGCGGTACT	41	6	C-21	AATCGGGCTG	36.9	4	S3	$(AG)_8T$	52	5
C-4	TGGCCCTCAC	41	5	C-22	GAAACGGGTG	36.9	0	S4	$(AG)_8C$	52	3
C-5	CATTCGAGCC	36.9	6	C-23	GTGATCGCAG	36.9	0	S5	$(AC)_8TT$	54	7
C-6	GTGTCGCGAG	41	6	C-24	TTCCGAACCC	36.9	3	S6	$(AC)_8AG$	55	4
C-7	TCACGTCCAC	36.9	4	C-25	GACCGCTTGT	36.9	8	S7	$(AC)_8CG$	58	6
C-8	AGGGCCGTCT	41	0	C-26	CAAACGTCGG	36.9	3	S8	$(AC)_8CT$	56	2
C-9	GGGTAACGCC	41	8	C-27	GTTGCCAGCC	41	4	S9	$(TC)_8A$	55	4
C-10	CCAGATGCAC	36.9	5	C-28	GGGAATTCGG	36.9	3	S10	$(TC)_8C$	55	6
C-11	ACGCAGGCAC	41	7	C-29	TGCTGCAGGT	36.9	5	S11	$(CA)_8G$	55	4
C-12	ACGATGAGCC	36.9	4	C-30	GGAGAGACTC	36.9	2	S12	$(CA)_8GG$	56	7
C-13	TGCGGCTGAG	41	8	C-31	AAGCCCGAGG	41	5	S13	$(GT)_8A$	55	5
C-14	ACGGCGTATG	36.9	7	C-32	CACCCGGATG	41	0	S14	$(GA)_8T$	55	5
C-15	CTACGCTCAC	36.9	6	C-33	GTCTCCGCAA	36.9	4	S15	$(GA)_8C$	55	7
C-16	TGCCGAGCTG	41	0	C-34	CTCCTGCCAA	36.9	5	S16	$(CGA)_5$	54	6
C-17	TGCGCCCTTC	41	4	C-35	ACCAGGGGCA	41	3	S17	$(ACA)_5$	44	3
C-18	AAAGCTGCGG	36.9	3	C-36	GGTTACTGCC	36.9	7	S18	$(CCG)_6$	72	5

conditions for RAPD analyses were consisted of an initial denaturation at 93℃ for 4 min followed by 40 cycles of 45 s denaturation at 94℃, 45 s annealing at 33℃ and 80 s extension at 72℃ with a final extension of 72℃ for 5 min using a thermal cycler (MJ Mini, Bio-Rad, USA).

In the case of ISSR primers, optimal annealing temperature was found to vary according to the base composition of the primers. ISSR amplifications were performed in a volume of 25 µl containing 25 ng of genomic DNA as template, 2.5 µl of 10× PCR buffer (1.5 mM $MgCl_2$), 200 µM dNTPs, 1 unit (U) of Taq DNA polymerase (Takara Shuzo Co.) and 1 µM of each primer. PCR was performed at an initial denaturation of 94℃ for 5 min followed by 35 cycles of 45 s denaturation at 94℃, 30 s at the annealing temperature and 90 s extension at 72℃ with a final extension at 72℃ for 5 min using a thermal cycler (MJ Mini, Bio-Rad, USA). The annealing temperature was adjusted according to the (melting temperature) Tm of the primer used in the reaction. The amplification products were electrophoresed in 0.8% agarose gels in 0.5×TBE (Tris-borate-EDTA) buffer. The size of the amplification products was estimated using a DNA ladder DL 2000 (Tiangen, China). The gels were photographed under UV light. Amplification with each primer was repeated twice to confirm reproducibility of the results.

Flow cytometry analysis

For flow cytometric analysis, young leaves of the target species (mother plant and plantlets growing *in vitro*) and of the internal standard (0.5 cm^2) were chopped simultaneously with a sharp razor blade in a plastic Petri dish with 1 cm^3 nucleus-isolation buffer [0.1 M Tris, 2.5 mM $MgCl_2$. $6H_2O$, 85 mM NaCl, 0.1 % (v/v) TritonX-100, 1 % (v/v) PVP-10; pH 7.0], incubated for 30 s, filtered through a 35 µm mesh and stained with 0.2 cm^3 staining solution including PI and RNase. The relative fluorescence intensities of the stained nuclei were measured by a flow cytometer (BD FACSCalibur). *Solanum lycopersicum cv. Stupicke* nuclei (2C= 1.95 pg, Borchert et al., 2007; Xing et al., 2010) was used as an internal standard for genome size. The absolute DNA amounts of the samples were calculated based on the values of the G_1 peak of *G. bicolor* and tomato. Routinely, at least 5000 nuclei were measured per sample.

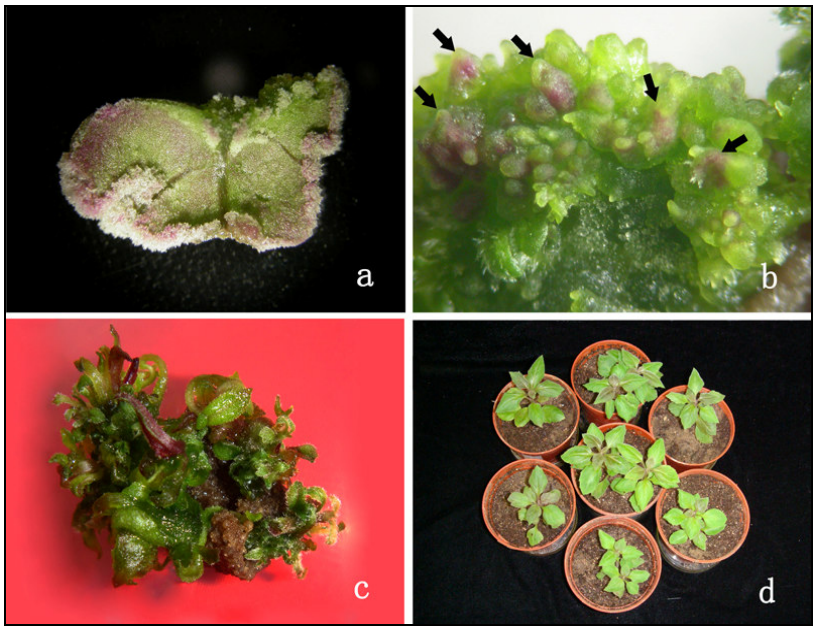

Figure 1. Adventitious shoot development and plant establishment in *G. bicolor.* (a) Purple callus grown on MS medium containing 2.0 mg/l 2,4-D + 0.5 mg/l BA after 2 weeks of culture; (b) shoot buds with purple color derived from the explants on MS + 0.5 mg/l TDZ + 4 mg/l $AgNO_3$ after 12 weeks of culture (callus induction medium was MS + 2.0 mg/l 2,4-D + 0.5 mg/l BA); Arrowhead denotes the vegetative buds; (c) multiple shoots formed by explants on MS + 0.5 mg/l TDZ + 4 mg/l $AgNO_3$ after 15 weeks of culture (callus induction medium was MS + 2.0 mg/l 2,4-D + 0.5 mg/l BA); (d) regeneration plants of *G. bicolor* transplanted in pots.

The 2C nuclear DNA content of unknown sample was calculated as follows:

$$\text{Sample 2C relative DNA content} = \frac{\text{Sample peak mean}}{\text{Standard peak mean}} \times \text{2C DNA content of a standard} \quad (2)$$

Statistical analysis

The experiments were set up in a completely randomized design. All values obtained from the three repeat experiments were averaged.The data were analyzed in relation to the variance and presented as mean ± standard error (SE). Statistical analysis of quantitative data was carried out using LSD test. All statistical analyses were performed at 5% level using DPS (version 3.01) (Ruifeng Info Technology Ltd., Hangzhou, China).

RESULTS AND DISCUSSION

Effect of plant growth regulators on callus and multiple shoot bud formation

The responses of the explants to various combinations of growth regulators on callus and adventitious shoot formation are summarized in Table 1. In our study, the frequency of callus formation was 100%. Callus formation was observed on the subepidermal area after 12 days of culture. The formed callus had purple color (Figure 1a), which indicated that it contained anthocyanins. Callus browning was observed after four weeks of culture and less brownlization was occurred in the medium containing 2,4-D. The 2,4-D was effective in resulting in a decrease of the frequency of callus browning (3.3 to 36.7%), whereas NAA was less efficient in browning control with the frequency of callus browning (66.7 to 68.3%) (Table 1).

There was a distinct difference in appearance of explants grown on shoot induction medium supplemented with $AgNO_3$. This study showed that the addition of $AgNO_3$ at 4 mg/l was beneficial in the alleviation of callus browning and greatly increased the number of plantlets produced. The browning was delayed and the callus browning rate was 31.6% on the shoot induction medium supplemented with $AgNO_3$, whereas the callus browning rate was 81.7% on the shoot induction medium without $AgNO_3$. The highest frequency of shoot regeneration (78.3%) was obtained in the medium containing 4 mg/l $AgNO_3$ and the shoot regeneration rate was only 43.3% for the control (without $AgNO_3$). Similarly, the highest number of shoots per explant (11.2) was obtained in the medium containing 4 mg/l $AgNO_3$, whereas the number of shoots per explant was only 5.1 for the control (without $AgNO_3$).

The presence of $AgNO_3$ in the shoot induction medium

not only enhanced shoot regeneration efficiency, but also expedited the initiation of adventitious buds; the multiple shoot initiated after 12 weeks on the shoot induction medium containing $AgNO_3$, were 3 to 4 weeks shorter than those without $AgNO_3$. As a result, 4 mg/l $AgNO_3$ was added to the shoot induction medium in all the subsequent experiments.

The increase in shoot regeneration frequency induced by $AgNO_3$ may be due to the interruption of the ethylene signal transduction pathway. $AgNO_3$ has been shown to promote regeneration by acting as a potent inhibitor of ethylene action in chilli (Ashrafuzzaman et al., 2009), cotton (Divya et al., 2008) and common bean (Dang and Wei, 2009).

Multiple shoot formation started to be evident after 12 weeks (Figure 1b). The number of explants with shoot buds was scored after 15 weeks culture and the results showed that 6-Benzylaminopurine (BA) stimulated multiplication to a higher extent compared to kinetin (KT). It was concluded that the promotive effect of BA on shoot regeneration resulted to higher frequency of regenerating explants. The addition of 2,4-D had a positive effect on shoot formation. The combination of BA and 2,4-D resulted in a higher proliferation rate compared with other combinations. Although four combinations of 2,4-D and BA were good and did not show any differences statistically, but the best SFC index (8.6) was obtained in the combination of 2,4-D at 2.0 mg/l and BA at 0.5 mg/l and was 1.8 to 2.8 times higher than those in the other combinations. The frequency of regenerating explants was 78.3% in the best combination. The number of shoots per explant was over 10 shoots per explant in most growth regulator combinations (Figure 1c, Table 1).

The SFC index is a parameter that is effective in evaluating the overall regenerative potential of explants, as it results from the combination of their regeneration frequencies with the number of shoots formed per explant (Ozudogru et al., 2005). Our results also showed that the higher shoot regeneration efficiency resulted from higher frequency of regenerating explants rather than the higher number of shoots per regenerating callus.

Rooting and plant establishment

A mixed pool of shoots (2.0 cm long or more) on shoot elongation medium was evaluated for rooting. Isolated shoots were excised and rooted and 100% rooting was achieved in IBA at 0.5 mg/l within 11 days of incubation. Acclimatization of rooted shoots was easily obtained after their transfer to pots (Figure 1d). Survival rate was over 80% and after seven weeks, rooted plantlets were transferred successfully to the greenhouse.

Genetic stability of regenerants

In order to confirm genetic integrity, the DNA of 40 random-selected regenerated plants was compared with the DNA of the mother plant. The 36 RAPD primers used in this analysis gave rise to 150 scorable band classes, ranging from 150 bp to 2.5 kb in size. The number of bands for each primer varied from 2 (C-30) to 8 (C-13), with an average of 4.17 bands per RAPD primer (Table 2). Of the 28 arbitrary ISSR primers initially screened, 18 produced clear and scorable bands (Table 2), ranging from 200 bp to 2.7 kb in size. The screening with the 18 ISSR primers generated 89 scorable band classes. The number of bands produced by each primer ranged from 2 to 7, with an average of 3.18 bands per ISSR primer. A total number of 9799 bands (numbers of plantlets analyzed × number of bands obtained with RAPD and ISSR primers) were generated, giving rise to monomorphic bands comparing all the regenerated plants and the mother plant. Examples of the monomorphic band classes obtained are shown in Figure 2a for RAPD markers and Figure 2b for ISSR markers. These analyses clearly indicated that there were no polymorphic bands.

Both RAPD and ISSR markers have been successfully applied to detect the genetic similarities or dissimilarities in various plants (Chandrika and Ravishankar Rai, 2009; Sikdar et al., 2010). RAPD and ISSR were chosen because they amplify different regions of the genome allowing better analysis of genetic stability/variation of regenerated plants, as well as their simplicity and cost effectiveness. When analyzing micropropagated plants of kiwifruit, Palombi and Damiano (2002) also suggested the use of more than one DNA amplification technique as advantageous in evaluating somaclonal variation.

Nuclear DNA content (genome size) is a specific karyological feature that is useful for systematic purposes and evolutionary considerations (Bennett and Leitch, 1995). Genome size is positively correlated to nuclear volume, cell volume, mitotic cycle time and the duration of meiosis. Tissue culture was considered as a key origin for chromosome instabilities, although the molecular mode of action is still unknown (Guo et al., 2005). *In vitro* regenerated *Hypericum perforatum* plants and their progenies showed cytological variations (Brutovska et al., 1998) potentially linked to high variation in the concentrations of characteristic bioactive compounds of the medicinal plant (Cellárová et al., 1994, 1997). Flow cytometry has aided this research as it has been demonstrated to be a convenient, accurate, rapid and highly reproducible method for estimating the nuclear genome size of plants.

In order to test the genetic variability of the regenerated protocol, leaves of the regenerated plants were subjected to flow cytometric measurements with tomato leaves (*S. lycopersicum cv. Stupicke*) as an internal standard and the absolute DNA contents of 30 regenerated plants of *G. bicolor* were calculated. The nuclear DNA contents of 30 regenerated plants varied from 15.07 to 15.17 pg/2C and it was similar to that of the mother plant (15.13 pg), the source material. This result showed no significant

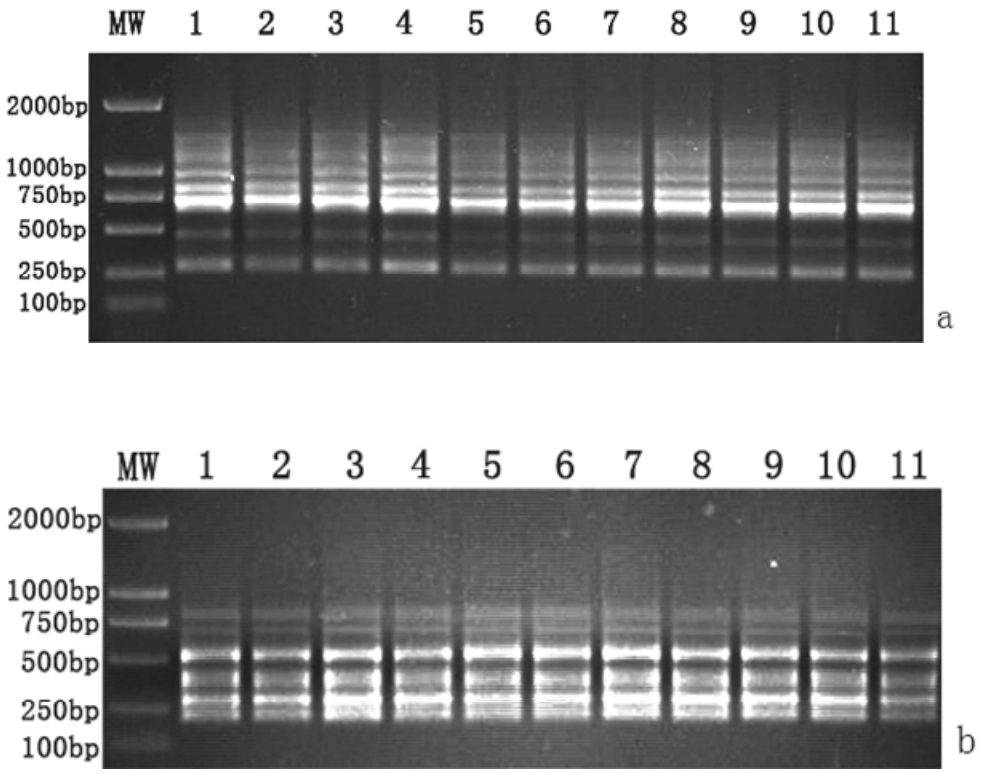

Figure 2. Analysis of genetic stability of the regenerated plants by RAPD and ISSR markers. (a) RAPD profile of plantlets regenerated from *G. bicolor* obtained with the primer C-25. Lanes 1, mother plant; lanes 2 to 11, regenerated plantlets. MW, DNA molecular size marker; (b) ISSR profile of plantlets regenerated from *G. bicolor* obtained with the primer S-2. Lanes 1, mother plant; lanes 2 to 11, regenerated plantlets.

differences (ANOVA, Tukey-HSD) between the mother plant and the *in vitro* cultured plants, which is indicative that they maintain their genetic stability during *in vitro* culture. This confirms the usefulness of tissue culture for the production of certified plant material to obtain herbal medicines.

In conclusion, we have successfully developed a novel and efficient protocol for plant regeneration of *G. bicolor* and analyzed the genetic stability of the regenerated plants by flow cytometry analysis, RAPD and ISSR molecular markers. This protocol will be useful for further secondary metabolic product, transformation and breeding studies.

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