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BIOTECHNOLOGY

Field performance evaluation and genetic integrity assessment in *Argyranthemum* 'Yellow Empire' plants recovered from cryopreserved shoot tips

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Abstract Field performance evaluation and genetic integrity assessment were conducted in *Argyranthemum* plants derived from cryopreserved shoot tips. Some variations in root formation and vegetative growth were found in the plants following cryopreservation, but morphologies of the leaves and flowers, and color, number and size of the flowers remained unchanged in the plants recovered from cryopreservation, compared with the control. Assessments of genetic integrity by the two molecular markers: inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) did not detect any polymorphic bands across the plants tested following cryopreservation. These data indicate that cryopreservation reduces, to a certain degree, root formation and vegetative growth, but it does not alter morphologies of leaves and flowers, may not cause any genetic alternations, and has no adverse effects on quantity and quality of the flowers. Therefore, the droplet vitrification cryopreservation can be considered promising for long-term preservation of *Argyranthemum* germplasm.

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Introduction

Argyranthemum, commonly called Marguerite (Walter *et al.* 2008), is a perennial ornamental plant, native to the Canary Islands, Spain, and Madeira, Portugal (Brickell 1999). In Norway, *Argyranthemum* is an economically important ornamental crop, with approximately 4.5 million plants produced per year.

The demand for novel flower cultivars by the market is stronger than ever, and the global flower industry thrives on novelty (Tanaka *et al.* 2005). Conservation and availability of genetic resources provide basic supports for breeding novel ornamental cultivars by both traditional and genetic engineering programs (Wang and Perl 2006; Kulus and Zalewska 2014). Cryopreservation, with several distinct advantages such as the capability for long-term storage, minimal requirement for storage space, and maintenance of genetic integrity of stored materials, is considered an ideal means for long-term conservation of plant genetic resources (Benson 2014; Wang *et al.* 2014).

Shoot tips are a preferred tissue over seeds, embryos, cells, and callus for conservation of plant genetic resources, because they are genetically identical to the mother plants (Engelmann 1997). Genetic integrity in the plants recovered from cryopreserved shoot tips is still a critical concern (Kulus and Zalewska 2014; Wang *et al.* 2014). To date, there have been numerous studies on assessments of genetic integrity in the plants recovered from cryopreserved shoot tips (Harding 2004; Wang *et al.* 2014). Inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) were among the frequently used tools for this purpose

(Harding 2004; Wang *et al.* 2014). For assessments of genetic integrity, most of the previous studies concentrated on tuber crops, forest species, and fruit plants (Harding 2004; Wang *et al.* 2014), while only a few used ornamental crops such as *Chrysanthemum* × *morifolium* (Martín and González-Benito 2005; Martín *et al.* 2011) and *Paeonia lactiflora* (Seo *et al.* 2007).

Evaluations of field performance in the plants regenerated from cryopreserved shoot tips are also important, but up to now, studies on this issue are quite limited, particularly in ornamental crops (Wang and Perl 2006; Kulus and Zalewska 2014). Furthermore, assessments of genetic integrity and evaluations of field performance in plants regenerated from cryogenic treatments were rarely included in the same study (Kaity *et al.* 2009; Ashmore *et al.* 2011).

We previously reported a droplet vitrification cryopreservation for shoot tips of *Argyranthemum* 'Yellow Empire' (Zhang *et al.* 2014). The current study focused on field performance evaluation and genetic integrity assessment in greenhouse-grown plants recovered from cryopreserved shoots tips of this plant.

Materials and Methods

Plant materials. *Argyranthemum* 'Yellow Empire', one of the most popular cultivars, in North European countries including Norway, was used in the present study. *In vitro* shoot cultures were established, according to Zhang *et al.* (2014), and maintained on a basic medium (BM) at 23°C under 18-h photoperiod with a light intensity of 50 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes (Philips TL-D Super 80, 58 W/840). BM was composed of MS (Murashige and Skoog 1962) medium, supplemented with 3% (*w*/*v*) sucrose and 0.6% (*w*/*v*) agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. Subculture was done once every 5 wk.

Cryopreservation. Cryopreservation of shoot tips by droplet vitrification was conducted, as described by Zhang *et al.* (2014). In brief, shoot tips (about 2.5 mm in length) containing five- to six-leaf primordia excised from 5-wk-old *in vitro* stock shoots were precultured overnight on BM supplemented with 0.5 M sucrose. Precultured shoot tips were treated for 20 min with a loading solution composed of MS medium containing 2 M glycerol and 0.4 M sucrose, followed by dehydration with plant vitrification solution 2 (PVS2, Sakai *et al.* 1990) for 30 min at 0°C. Afterward, each dehydrated shoot tip was transferred onto 2.5 μL PVS2 carried on sterile aluminum foil strip (0.7×2 cm), prior to a direct immerge into liquid nitrogen (LN) for 1 h. Rewarming was performed by removing the aluminum strips from LN and quickly plunging into an unloading solution composed of 1.2 M sucrose in MS for 20 min at room temperature. Cryopreserved shoot tips were post-cultured on a recovery medium composed of BM supplemented with 0.05 mg L^{-1} gibberellic acid 3 (GA_3), for shoot regrowth. The cultures were grown in the dark for the first 3 d and then transferred onto the same fresh recovery medium under light conditions, as described for the *in vitro* stock cultures. Shoots (≥0.5 cm in length) regenerated directly without any callus formation after 2 mo of culture on the recovery medium. Regenerated shoots were transferred to BM for further growth for 6 mo (subculture every 5 wk), in order to recover and stabilize after cryopreservation.

Root formation. Shoots (longer than 2 cm), which were regenerated from cryopreserved shoot tips and *in vitro* cultures, were removed from BM, washed thoroughly with tap water to remove the agar, and then rooted in Jiffy-7 peat pellets (Norgro AS, Hamar, Norway) contained in black plastic tray. The cultures were covered with white plastic bags to maintain high humidity and prevent the shoots from wilting and placed in greenhouse conditions under 8-h photoperiod with a supplementary light intensity of 150 μmol m^{-2} s^{-1} provided by high-pressure sodium (HPS) lamps (400 W, GAN 4-550, Norway) at a consistent temperature of 22±2°C. The bags were gradually uncovered to reduce the humidity and removed totally after 2 wk of rooting. Data on rooting percentage, number of roots (≥0.5 cm in length), and length of the longest root per shoot were recorded after 3 wk of rooting.

Vegetative and reproductive growth. After 3 wk of rooting, the plants with well-developed roots were transferred into 6-cm pots containing peat (Degernes Torvstrøfabrikk, Degernes, Norway) for vegetative growth, under the same greenhouse conditions, as used for rooting. Parameters including plant height, number of fully opened leaves per plant, and node number and length were measured after 4 wk of growth. The plants were then transferred into 12-cm pots containing peat, for further vegetative growth and flower production, under 18-h photoperiod with a supplementary light intensity of 200 μmol m^{-2} s^{-1} at a consistent temperature of 22±2°C. The plants were pruned to maintain eight leaves per plant to induce growth of lateral shoots. Data on vegetative growth and flower production were recorded after 8 and 12 wk of growth, respectively.

Assessment of genetic integrity. Plants regenerated from cryopreserved shoot tips, which had been grown in peat under greenhouse conditions with an 18-h photoperiod for 8 wk, were used for assessment of genetic integrity by ISSR and AFLP markers. Genomic DNA was extracted from 100-mg fresh leaf tissue using a DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Purified total DNA was quantified and its quality verified by ultraviolet spectrophotometry.

ISSR analysis. Forty ISSR primers were screened to select suitable primers for assessment of genetic stability in the greenhouse-grown plants. PCR was performed in a 25-μL reaction solution containing 2.5 μL 10×PCR buffer, 0.2 μL (1 U) Taq polymerase (Roche, Indiana, IN), 0.5 μL dNTP (10 mM), 0.5 μL primer (100 μM), and 1 μL template DNA (100 ng/μL). DNA amplification was performed in a PCR instrument (Bio-Rad, Singapore, Singapore) using the following reaction conditions: initial denaturation step at 94°C for 2 min; followed by 40 cycles at 94°C for 30 s, different annealing temperature (42°C for ISSR-ARG-10, 44°C for ISSR-ARG-11, and 52.5°C for other primers, see Table 4) for 45 s, and 72°C for 2 min; followed by a final extension step at 72°C for 7 min. The PCR products were separated by electrophoresis in 2% (*w*/*v*) agarose gel containing 0.1% (*w*/*v*) ethidium bromide and visualized under ultraviolet light. The molecular 100-bp and 1-kb DNA ladder (New England BioLabs Inc, Ipswich, UK) were used for estimating the size of the amplified products.

ISSR fingerprints were manually scored for the presence (1) and absence (0) of each band. Bands of equal molecular weight and mobility generated by the same primer were considered to represent the same locus. Both distinct monomorphic bands and polymorphic bands were scored. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and were not scored.

AFLP analysis. AFLP was performed as described by Elameen *et al.* (2008), with modifications that used fluorescently labeled primers instead of radioactive ones. Briefly, genomic DNA (300 ng) was double-digested with *Eco*RI and the *Mse*I isoschizomer *Tru1*I. Following ligation of the restriction fragments to the adaptors, pre-amplification PCR was carried out with non-selective primers in a total volume of 25 μL, containing 5 μL of five-fold diluted ligation product. Fluorescent primers were labeled, according to Dresler-Nurmi *et al.* (2000). The *Eco*RI primers were labeled blue with 5′-6-acrylamide carboxy fluorescein (6-FAM), and *Mse*I primers were labeled yellow 5′-4,7,2,4,5,7 hexachloro-6-carboxy fluorescein (HEX).

The fluorescently labeled PCR products were analyzed using an ABI3730 DNA Analyzer. One microliter of PCR products was added to a loading buffer containing 8.75 μL Hi-Di formamide (Applied Biosystems, Foster City, CA) and 0.25 μL of GeneScan 500 LIZ size standard (Applied Biosystems). The data was collected using the software Data Collection v2.0 (Applied Biosystems), while GeneMapper v4.1 (Applied Biosystems) was used to derive the fragment length of the labeled DNA fragments using the known fragment lengths of the LIZ-labeled marker peaks.

AFLP bands were recorded manually and scored for presence (1) or absence (0). The genetic similarity was estimated with the Dice's coefficient (Dice 1945) and Jaccard's coefficient (Jaccard 1908). Both analyses resulted in the same results, and only the results obtained by the Dice's coefficient are presented. The matrix of similarity data was analyzed using unweighted pair group method with arithmetic mean (UPGMA), as suggested by Sneath and Sokal (1973). UPGMA clustering was also carried out for all the plants tested according to the treatments: cryopreserved and non-cryopreserved (control). These analyses were performed using NTSYSpc software (Rohlf 2000).

Experimental design and statistical analysis of data. All experiments of field performance were organized based on a complete random design. Shoots regenerated from i*n vitro* culture without cryopreservation served as the control. At least ten samples were used in each treatment of three replicates, and each experiment was repeated twice. Data were analyzed by Student's *t* test ($P \leq 0.05$). Thirty plants recovered from cryopreservation and 30 from *in vitro* cultures (the control) were randomly selected, from a population of 150 plants following cryopreservation and 200 *in vitro* culture-derived plants, respectively, and used in the experiments of assessments of genetic integrity. The experiments of ISSR and AFLP were repeated twice to confirm their repeatability.

Results

Root formation. Roots were easily observed on the outside surface of Jiffy-7 after 1 wk of rooting, with a good root system developed after 3 wk of rooting in the shoots regenerated from cryopreserved shoot tips and *in vitro* cultures (the control; Fig. 1*A*). No significant differences were found in rooting percentage between the shoots regenerated from cryopreserved shoot tips (95%) and the control (100%; Table 1). However, root number and length of the longest root were greater in *in vitro*-derived shoots (14.9 and 4.4 cm, respectively) than in cryo-derived shoots (12.0 and 3.7 cm; Table 1).

Vegetative growth. After 4 wk of growth, under 8-h photoperiod, plant height was similar in plants regenerated from cryopreserved shoot tips (9.1 cm) and the control (9.2 cm; Table 2). However, there were significant differences in other parameters including number of fully opened leaves, and node number and length between the two types of plants (Table 2). Morphologies of leaves were identical between cryopreserved and control shoot tips (Fig. 1*B*). After 8 wk of growth under 18-h photoperiod, plant height and number, and length of lateral shoots were significantly higher in plants regenerated from control than from cryopreserved shoot tips (Table 2 and Fig. 1*D*), but no differences were found in diameter of the main stem (Table 2). After 12 wk of growth under 18-h photoperiod, morphologies of leaves (Fig. 1*C*) were identical in

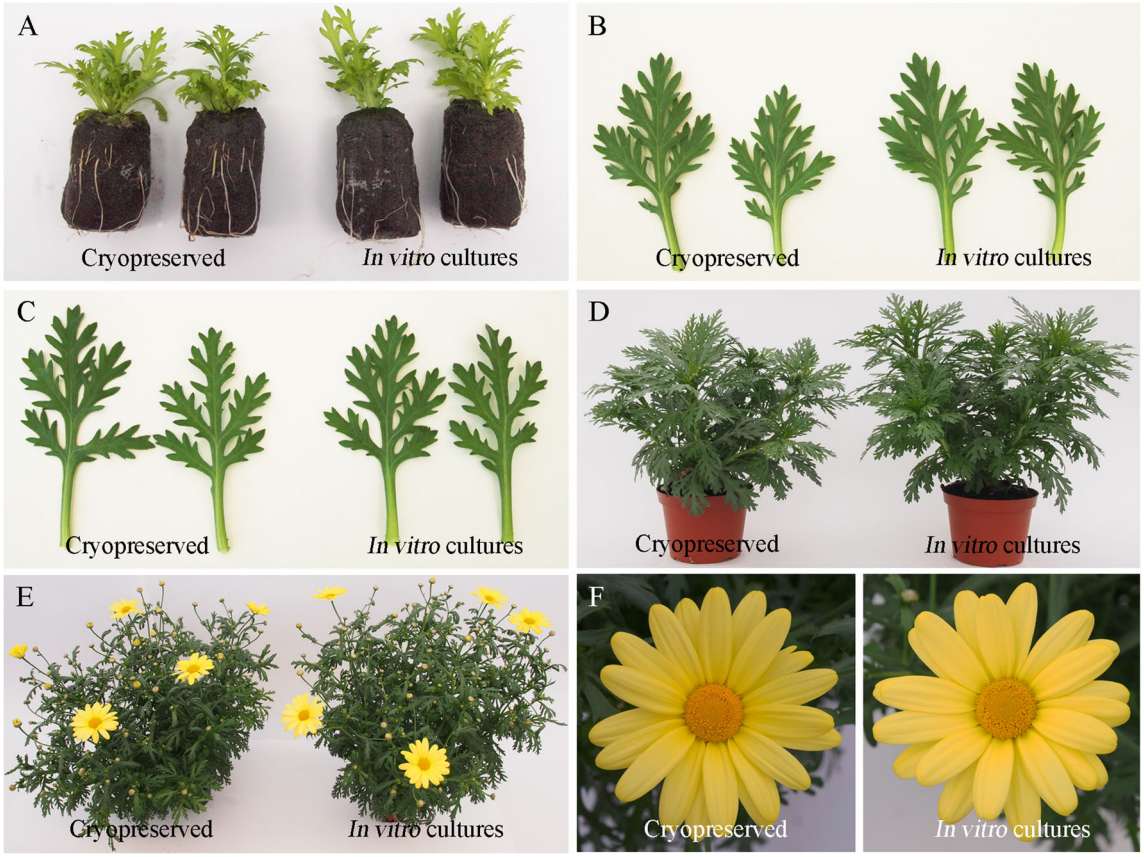

Figure 1. Root formation and field performance of plants regenerated from cryopreserved shoot tips and *in vitro* cultures of *Argyranthemum* 'Yellow Empire'. (*A*) Root formation in shoots regenerated from cryopreserved shoot tips and *in vitro* cultures after 3 wk of rooting. Morphologies of leaves of plants regenerated from cryopreserved shoot tips and *in vitro* cultures after 4 wk of vegetative growth under 8-h photoperiod (*B*) and 12 wk under 18-h photoperiod (*C*) under greenhouse conditions. (*D*) Vegetative growth in plants regenerated from cryopreserved shoot tips and *in vitro* cultures after 8 wk of growth under 18-h photoperiod under greenhouse conditions. Plant growth (*E*) and morphologies of flowers (*F*) of plants regenerated from cryopreserved shoot tips and *in vitro* cultures after 12 wk of growth under 18-h photoperiod under greenhouse conditions.

the two types of plants. The general appearance of the plants was similar between the two treatments (Fig. 1*E*), although plant height and biomass of the plants measured by fresh and dry weight were significantly smaller in cryo-derived plants than *in vitro* culture-derived ones (Table 3).

Flower production. The number and diameter of flowers were similar in the plants regenerated from cryopreserved shoot tips and *in vitro* cultures (Tables 2 and 3). Morphologies and color of the flowers were identical between them (Fig. 1*F*).

ISSR analysis. Out of the 40 primers tested in the ISSR analysis, ten produced strong and clear reproducible bands (Table 4). These ten primers resulted in 50 clear bands for each plant. The number of bands produced by each primer varied from four to eight (Table 4). In total, 1500 bands were scored across the 30 plants analyzed. No polymorphic bands were detected in all plants regenerated from cryopreserved shoot tips and the control (Table 4 and Fig. 2).

AFLP analysis. AFLP analysis of all 30 plants using five primer combinations (Table 5) resulted in a total number of 151 clear monomorphic bands (Fig. 3). The number of monomorphic bands per primer combination ranged from 26 to 34 bands, with an average of 30.2 monomorphic bands per primer combination. No polymorphic bands were detected across

Table 1. A comparison of root formation in shoots regenerated from cryopreserved shoot tips (+LN) and *in vitro* cultures (control) of *Argyranthemum* 'Yellow Empire'

Shoots regenerated from	Root formation (%)	No. of roots per shoot	Longest root (cm)
+LN	95.0±0.6 a	12.0±1.2 a	3.7±0.2 a
Control	100 a	14.9±0.9 a	4.4±0.2 b

Results were recorded after 3 wk of rooting. Data were presented as means±SE and with different *letters* in the same *column* indicate significant differences at $P \leq 0.05$ by the Student's *t* test ($n=60$)

Table 2. A comparison of vegetative growth in plants regenerated from cryopreserved shoot tips (+LN) and *in vitro* cultures (control) of *Argyranthemum* 'Yellow Empire'

Plant recovered from	4-wk growth, 8-h photoperiod				8-wk growth, 18-h photoperiod				
	Plant height (cm)	Fully opened leaves	No. of nodes	Node Length (cm)	Plant height (cm)	Main stem dia. (cm)	No. of lateral shoot	Shoot length (cm)	No. of flower buds
+LN	9.1±0.2 a	17.9±0.5 a	6.3±0.2 a	1.5±0.1 a	19.6±0.4 a	1.1±0.1 a	6.8±0.3 a	18.7±0.4 a	4.4±0.2 a
Control	9.2±0.3 a	16.8±0.3 b	5.6±0.2 b	1.7±0.1 b	21.9±0.4 b	1.1±0.1 a	8.3±0.2 b	19.8±0.4 b	4.6±0.2 a

Data were presented as means±SE and with different *letters* in the same *column* indicate significant differences at $P \leq 0.05$ by the Student's *t* test ($n=60$)

Table 3. A comparison of vegetative growth and flower production in plants regenerated from cryopreserved shoot tips (+LN) and *in vitro* cultures (control) of *Argyranthemum* 'Yellow Empire'

Parameters tested	Plant regenerated from	
	+LN	Control
Plant height (cm)	58.7±0.6 a	61.2±0.5 b
Number of flowers	6.7±0.7 a	8.3±0.6 a
Diameter of flowers (cm)	6.9±0.4 a	7.4±0.1 a
Fresh weight of plants (g)	503.0±21.0 a	565.1±12.0 b
Dry weight of plants (g)	49.2±2.0 a	54.7±1.3 b

Results were recorded after 12 wk of growth under 18-h photoperiod in greenhouse conditions. Data were presented as means±SE and with different *letters* in the same *column* indicate significant differences at $P \leq 0.05$ by the Student's *t* test ($n=60$)

all samples using these five primer combinations. The UPGMA analysis clustered all of the plants tested into one main group (data not shown), indicating no variations between cryo-derived and *in vitro* culture-derived plants. The matrix of similarity data showed no variations between plants regenerated from cryopreserved shoot tips and the control (data not shown).

Discussion

Field performance and genetic integrity of *Argyranthemum* 'Yellow Empire' plants derived from cryopreserved shoot tips were compared with those from *in vitro* cultures (the control) in the present study. For field performance, although some differences were found in root formation and vegetative growth between the two types of the plants, morphologies of the leaves and flowers, and color, number and size of the flowers were identical to each other. For genetic integrity, no polymorphic bands were detected by ISSR and AFLP in the two types of the plants. These results indicate that cryopreservation reduces, to a certain degree, root formation and vegetative growth, but it does not alter morphologies of leaves and flowers, may not cause any genetic alternations, and has no adverse effects on quantity and quality of the flowers, the most economically important trait considered in production of ornamental crops. To the best of our knowledge, this is the first report investigating field performance and genetic integrity in ornamental crops following cryopreservation.

Although information on field performance evaluation of ornamental crops following cryopreservation has been quite limited (Wang and Perl 2006; Kulus and Zalewska 2014), there have been a number of studies on other crops. Harding and Staines (2001) found that *Solanum tuberosum* plants recovered from cryopreserved shoot tips showed a range of differences in height, tuber weight and size, and leaf morphologies compared to field-propagated plants. Medina *et al.*

Table 4. ISSR primer names, primer sequences, and numbers of amplified bands in plants regenerated from cryopreserved shoot tips of *Argyranthemum* 'Yellow Empire'

Primer name	Primer sequence (5′–3′)	Annealing temperature (°C)	No. of amplified bands	No. of polymorphic bands
ISSR-ARG-02	$(CAC\ ACA)_2$ CAC AG	52.5	5	0
ISSR-ARG-03	$(ACA\ CAC)_2$ ACA CT	52.5	4	0
ISSR-ARG-04	$(ACA\ CAC)_2$ ACA CC	52.5	6	0
ISSR-ARG-05	$(ACA\ CAC)_2$ ACA CG	52.5	4	0
ISSR-ARG-06	$(TGT\ GTG)_2$ TGT GG	52.5	6	0
ISSR-ARG-07	$(GAG\ AGA)_2$ GAG AYG	52.5	8	0
ISSR-ARG-09	$(TGT\ GTG)_2$ TGT GRT	52.5	5	0
ISSR-ARG-10	$(GACA)_4$	42.0	4	0
ISSR-ARG-11	$(GA)_9T$	44.0	4	0
ISSR-ARG-13	$(AGG)_6$	52.5	4	0
Total			50	0

(2007) found some differences in fruit quality parameters such as fruit shape, internal flesh color, fruit firmness, and °Brix between strawberry plants (*Fragaria* × *ananassa*) derived from cryopreserved shoot tips and *in vitro* cultures. However, these two types of plants produced similar results in marketable fruit yield, second-class fruit yield, and fruit weight. No adverse effect on agronomic traits appeared to be caused by cryopreservation. Similar results were also reported in other plant species such as *Dioscorea floribunda* (Ahuja *et al.* 2002) and *Carica papaya* (Kaity *et al.* 2009; Ashmore *et al.* 2011). These results agree with the present study.

Molecular markers have been widely used for assessment of genetic stability of regenerants derived from cryopreservation (Harding 2004; Wang *et al.* 2014). Using ISSR for assessment of the genetic stability of plants regenerated from cryopreserved shoot tips of *Malus* × *domestica*, Liu *et al.* (2008) did not detect any polymorphic bands between control and cryopreserved shoots. AFLP analysis revealed no genetic variations in cryopreserved plants of *Humulus lupulus* (Peredo *et al.* 2008). Maintenance of the genetic fidelity in the plants recovered from cryopreserved shoot tips was reported in a great number of plant species, such as *S. tuberosum* (Hirai and Sakai 1999), *M.* × *domestica* (Hao *et al.* 2001), *F.* × *ananassa* (Hao *et al.* 2002), *Populus tremula* × *Populus tremuloides* (Jokipii *et al.* 2004), *P. lactiflora* (Seo *et al.* 2007), *Dioscorea rotundata* (Mandal *et al.* 2008), *Hypericum perforatum* (Skyba *et al.* 2010), and *C. papaya* (Kaity *et al.* 2013). These data supported our findings that no polymorphic bands were detected by ISSR and AFLP in the greenhouse-grown plants derived from cryopreserved shoot tips.

Use of more than one DNA amplification technique, to amplify different regions of the genome, provides a better analysis of genetic variation than a single method (Martín *et al.* 2011). Assessment of genetic stability by RAPD in *Chrysanthemum* regenerants derived from cryopreserved shoot tips detected a polymorphic rate at 5.8%, while AFLP revealed 40.1% of genetic variations (Martín *et al.* 2011). The present study employed two molecular techniques: ISSR and AFLP. ISSR uses universal markers; is technically simple, quick to perform, and reproducible; and requires only small amounts of DNA (Bornet and Branchard 2001). AFLP has a higher multiplex ratio than other molecular markers, produces highly reproducible results, and allows direct analysis of variation at loci throughout the whole genome (Powell *et al.*

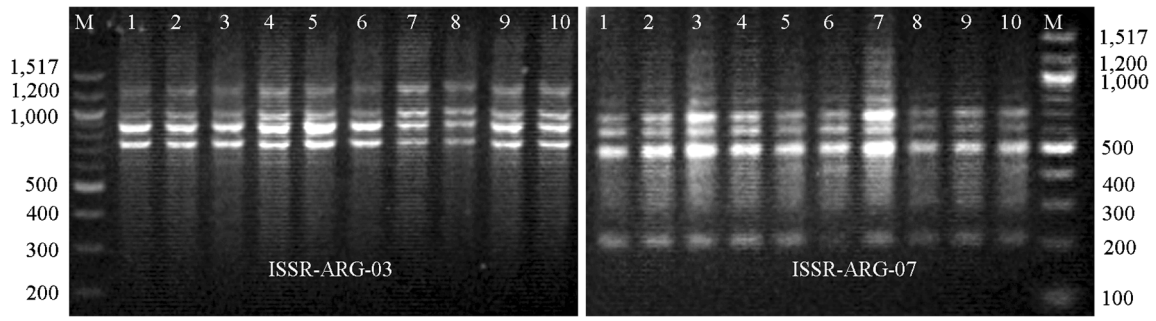

Figure 2. ISSR banding pattern in plants regenerated from *in vitro* cultures and cryopreserved shoot tips of *Argyranthemum* 'Yellow Empire'. *M* marker, *lanes 1–5* plants regenerated from *in vitro* cultures, *lanes 6–10* plants regenerated from cryopreserved shoot tips.

Table 5. Primer combinations used for amplification of AFLP analysis in plants regenerated from cryopreserved shoot tips of *Argyranthemum* 'Yellow Empire'

Primer combination	*Eco*RI primer sequences (5′–3′)	*Mse*I primer sequences (5′–3′)
E19 X M15	GAC-TGC-GTA-CCA-ATT-CGA	GAT-GAG-TCC-TGA-GTA-ACA
E19 X M16	GAC-TGC-GTA-CCA-ATT-CGA	GAT-GAG-TCC-TGA-GTA-ACC
E12 X M17	GAC-TGC-GTA-CCA-ATT-CAC	GAT-GAG-TCC-TGA-GTA-ACG
E12 X M16	GAC-TGC-GTA-CCA-ATT-CAC	GAT-GAG-TCC-TGA-GTA-ACC
E19 X M17	GAC-TGC-GTA-CCA-ATT-CGA	GAT-GAG-TCC-TGA-GTA-ACG

1996). Therefore, results of genetic stability assessments in the plants derived from cryopreserved shoot tips of *Argyranthemum* here should be considered reliable.

There were also some studies in which genetic variations assessed by molecular markers were found in the regenerants from cryopreserved shoot tips of plant species such as *Dendranthema grandiflora* (Minäno *et al.* 2009), *Rubus* (Castillo *et al.* 2010), *Chrysanthemum* × *morifolium* (Martín *et al.* 2011), and *Rabdosia rubescens* (Ai *et al.* 2012). In the case of *Rubus* (Castillo *et al.* 2010), when plants showing polymorphic bands were transferred to the field, they exhibited the same AFLP fingerprints as their original mother plants, indicating that there might be a transitory phase of the polymorphism. Similar results were also reported for *Abies cephalonica* (Aronen *et al.* 1999) and *C. papaya* (Kaity *et al.* 2013). Cryopreservation procedure involves not only freezing in LN but also tissue culture steps for preparing samples before cryopreservation and for plant regeneration after cryopreservation. Tissue culture procedures may also contribute to somaclonal variation (Harding 2004; Wang *et al.* 2014). Nevertheless, all these data suggest that genetic variation may occur throughout the cryopreservation process and/or the associated tissue culture steps (Harding 2004; Wang *et al.* 2014), and therefore, the genetic stability of the regenerants following cryopreservation must be assessed.

The present study shows that cryopreservation of *Argyranthemum* shoot tips does not lead to genetic variation or loss of field performance. Therefore, the droplet

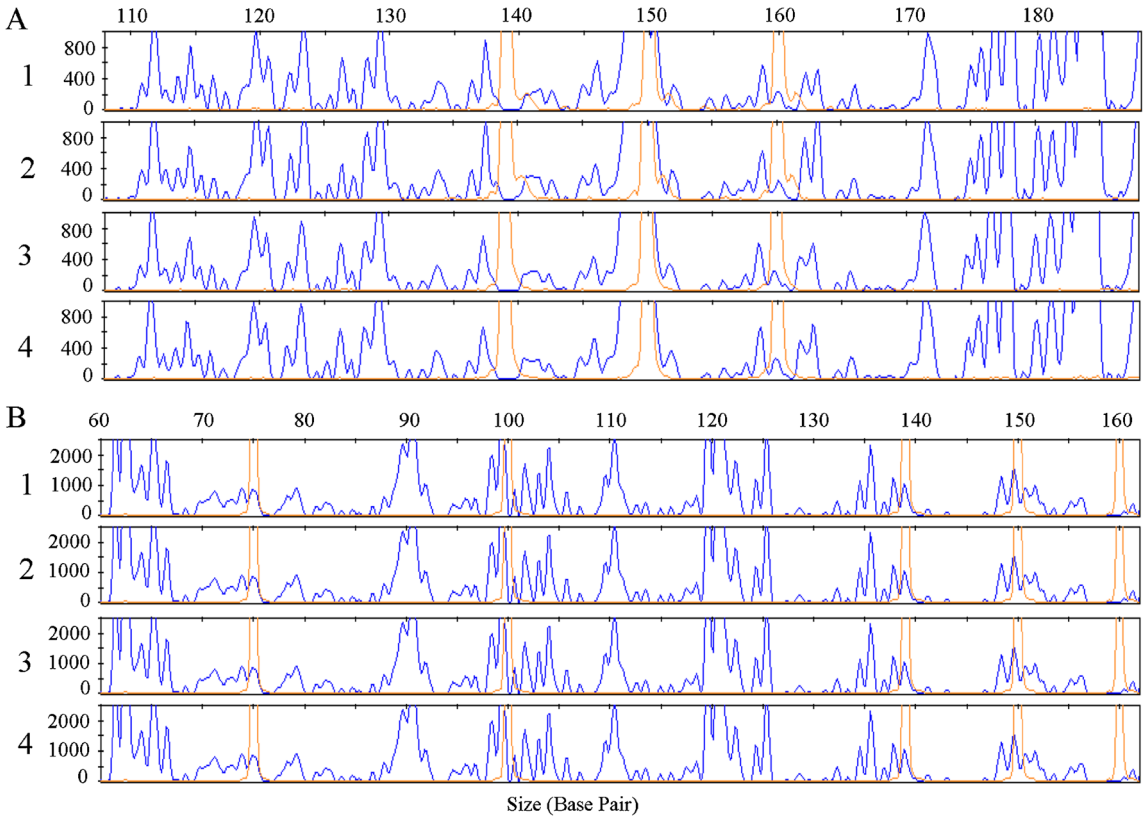

Figure 3. AFLP patterns (obtained with GeneMapper software) of plants regenerated from *in vitro* cultures and cryopreserved shoot tips of *Argyranthemum* 'Yellow Empire' after 8 wk of growth with long-day conditions under greenhouse. AFLP patterns obtained with primer E19 X M15 (*A*) and primer E19 X M16 (*B*). Numbers *1*, *2*, and *3* are plants regenerated from cryopreserved shoot tips and *4* from *in vitro* cultures (control).

vitrification cryopreservation developed here can be considered a promising tool for the long-term storage of *Argyranthemum* germplasm.

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