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Ex situ conservation using medium-term cultures in *Moehringia jankae* Griseb. ex Janka (Caryophyllales: Caryophyllaceae) and genetic stability assessment using ISSR

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Abstract: *Moehringia jankae* Griseb. ex Janka is a Balkan endemic plant which is spread in Romania and Bulgaria. Besides *in situ* conservation, a complementary *ex situ* conservation approach involves the elaboration of short and medium-term preservation protocols. For this purpose, several experimental variants consisted in the nutrients reduction or the supplementation of culture media with different chemical compounds as clormerquat, flurprymidol, abscisic acid and mannitol at different concentrations were tested and their efficiency for medium-term cultures establishment was evaluated. The results concerning growth reduction, regeneration and survival rate showed that this taxon can be efficiently medium-term preserved using 32 and 48 μM flurprymidol, mannitol at 0.16 M and clormerquat at 2.5 mM, this approach being a reliable and cheap method for *ex situ* preservation. From minimal cultures induced using factors with positive effect, after seven months well developed plants were obtained and evaluated concerning their genetic stability, using ISSR markers. Cultures established and maintained in the presence of abscisic acid, flurprymidol, mannitol or clormerquat did not affect significantly the stability of plants regenerated after seven months.

Key words*:* medium-term, conservation, flurprymidol, clormerquat.

Introduction

Moehringia jankae Griseb. ex Janka (Syn. *Arenaria jankae* (Griseb. ex Janka) Fernald) is a Balkan endemic plant which grows on rocky, dry places in SE of Romania (in Dobroudja-Tulcea and Constanta county) and in NE of Bulgaria (in Shumen district) with relatively limited and diffused populations. This taxon is considered vulnerable in the Red Book of Vascular Plants from Romania (Dihoru & Negrean 2009) and endangered according to the Red Data Book of Republic of Bulgaria (Peev 2015).

Biotechnological methods can help *in situ* measures providing an alternative for *ex situ* plant conservation (Engelmann 2011, Reed et al. 2011, Cruz et al. 2013, Pathak & Abido 2014; Rajasekharan & Sahiran 2015). Minimal cultures involving different methods of growth retardation has been widely used in the case of cultivated plants available for international exchanges (Cha-um & Kirdmanee 2007). To establish medium-term cultures, besides physical methods (low temperature storage, reduced illumination, small culture vessels and short photoperiod), different chemical methods were also applied alone or in combination. For inducing minimal *in vitro* cultures, different class of compounds were restricted or supplemented in the culture media as: the reduction of salts in the minimal media (Malik et al. 2005; Renau-Morata et

al. 2006; Garcia-Jimenez et al. 2006); the addition of ethylene inhibitors or gibberellic acid inhibitors as flurprymidol, paclobutrazol or ancymidol (Sharkar et al. 2001, Madubanya et al. 2006) or abscisic acid (Jarret & Gawell 1991; Malik et al. 2005; Renau-Morata et al. 2006). Also, were used osmotic agents as: polyethylene glycol (Holobiuc et al. 2014); sugar and sugar alcohols (Hao & Deng 2003; Charoensub et al. 2004; Divakaran et al. 2006)

In *M. jankae,* owing the positive *in vitro* response and the fast growth rate in the short-term culture, the elaboration of a protocol for growth reduction was necessary, this approach being a viable and cheaper alternative to *ex situ* preserve during prolonged time without important additional costs which can be applied to other related species.

This study had the aim to elaborate an efficient medium-term conservation protocol and to evaluate the genetic stability of the regenerants of the vulnerable taxon *Moehringia jankae* using ISSR markers.

Material and methods

Several experimental variants were tested for medium-term cultures establishment. After seven months of preservation in medium term culture, regenerated plants were evaluated using ISSR markers concerning their genetic stability.

Medium-term cultures induction*:* from primary cultures previously obtained consisted in well-developed plants, grown in large Phytatray vessels, double node explants (1 cm height) were excised and were cultured on 13 variants of solid MS basal medium (Murashige & Skoog 1962) gelified with 8 g/l (w/v) agar and supplemented with different chemical compounds to induce growth reduction. Five explants/culture vessel in five repetitions were cultured for each experimental variant. The cultures were made in glass jars of 55 mm diameter and 85 mm high, containing 30 ml of medium and grown in a cultivation chamber at 25 ±1°C with 16 hrs of cool and white fluorescent light at 60 μM m^{-2} s^{-1} per day. As growth limitation factors were used: abscisic acid, clormerquat, flurprymidol and mannitol.

Variant **V1**, consisted in MS medium added with 20g/l sucrose, used as control. **V2** contains MS macro salts reduced at ½, **V3**- MS + sucrose (su) reduced at 10 g/l. The other media variants contain 20 g/l sucrose. All media had pH adjusted at 5.8 before autoclaving under 118 kPa and 120°C for 20min.

V4-V13 variants contain retardation factors at different levels as follows: **V4**- MS+ 18.9 μM abscisic acid (ABA)**, V5**- MS + 37.8 μM ABA, **V6**-MS+ 1.26 mM clormerquat (Cycocel), **V7**- MS+ 2.5 mM clormerquat, **V8**-MS+16μM flurprymidol, **V9**-MS+ 32 μM flurprymidol, **V10**- MS+ 48 μM flurprymidol, **V11**- MS+ 64 μM flurprymidol, **V12**- MS+ 0.16 M mannitol, **V13**- MS+ 0.32 M mannitol.

The *in vitro* response of *M. jankae* during medium-term culture was evaluated after 1, 2, 3 and 6 months using several parameters: the maximum length (cm) of the best developed shoot/initial explant, the mean number of shoots/initial explants, the rooting rate (%) registered after different interval of time, the survival rate (%) registered after 6 months. After seven months of cultures, shoots induced in the presence of different growth retardants belonging to different initial clones were cultured on MS medium to obtain well-developed plants.

Statistical analysis: all data were analyzed using One-Way ANOVA test (at $p \leq 0.05$). The percent values were converted into arcsine x, prior statistical analysis. The significance of differences between experimental variants was assessed using a post-hoc test Bonferroni-Holmes at 95% confidence interval (Daniel's XL Toolbox version 6.60). The results were expressed as the mean ± standard deviation (SD).

Molecular analysis using ISSR- markers

Plants derived from two clones maintained in medium-term cultures in the presence of the best variants reduced -growth as 37.8 μM ABA (V5), 2.5 mM clormerquat (V7), 32 μM (V9) and 48 μM (V10) flurprymidol and 0.16 M mannitol (V12), were analyzed concerning their genetic stability. For each sample, total DNA was extracted from 100 mg of shoots.

Two clones of *M. jankae* were analyzed: **-clone 1** consisted in 30 samples: ten from control (1.1-1.10), ten derived from medium V12 (2.1-2.10) and ten derived from variant V5 (3.1-3.10). The **clone 2** included 40 samples: ten from control (4.1-4.10), ten from variant V7 (5.1-5.10), ten derived from variant V9 (6.1-6.10) and ten derived from variant V10 (7.1-7.10). Genomic DNA was extracted using NucleoSpin Plant II (Machererey-Nagel) Kit and DNA concentration was determined using BioDrop Duo. Seven ISSR primers like UBC (The University of British Columbia Biotechnology Laboratory, Canada) were tested, having between 16-18 bases provided by Dexter com, Romania (Table 1). The polymerase chain reaction (PCR) amplifications were performed in a 25 μl volume containing template DNA at concentration 50 ng/μl diluted with nuclease free water (Promega, USA), 2.5 μl of primer at concentration 10 μM and 12.5 μl

Table 1. The primers tested and the results for ISSR amplifications.

Primer cod	Secquence (5'-3')	T_m (°C)	Range of amplification (pb)	No of scorable bands per primer	No of polymorphic bands	Total no. of amplified bands	Polymorphic regenerats
MC1	ACA CAC ACA CAC ACA CG	53	250-2000	6(clone1)	-	-	-
MC2	ATG ATG ATG ATG ATG ATG	43	350-2000	8 (clone1) 6 (clone2)	4 3	179 231	1.1, 1.2, 3.2 4.3, 4.9,4.10, 5.1,6.2,7.9
MC3	GAG AGA GAG AGAGAG AYG	49	250-1650	6 (clone1) 7 (clone2)	0 0	180 280	0 0
MC4	GAC AGA CAG ACA GAC A	47	250-1000	9 (clone1) 8 (clone2)	5 2	179 304	1.1,1.2,1.3, 1.7,1.8,2.6, 2.8 7.1-7.10
MC5	GGA GAG GAG AGG AGA	48	250-2000	8 (clone1)	1	237	2.4, 2.10, 3.6
MC6	GAG AGA GAG AGA GAG AT	45	400-2000	6(clone1)	-	-	-
MC7	VHV GTG TGT GTG TGT GT	52	450-1000	7(clone1)			

Go taq Green Master Mix (Promega). The amplifications were made in an Eppendorf Mastercycler gradient (Germany).

The PCR was optimized by modifying the annealing temperature to melting point (T_m) for individual primers. The cycling conditions were: initial denaturation step 2 min at 94°C, followed by 30 cycles of 30 s at 94°C (denaturation), 30 s at specific annealing temperature (Table 1), 40 s at 72°C (extension), and 1 cycle for 5 min final extension step at 72°C. Electrophoretic separation was performed with 7 µl of amplified products on 1,5% agarose gel. DNA amplification bands were stained with ethidium bromide at 0.02µg/ml gel concentration. The weight molecular marker (Mg) used was 1Kb Plus DNA Ladder (Invitrogen). The PCR amplification for each ISSR primer was made in two repetitions, only clear and completely reproducible bands were included in data evaluation. The bands were scored as presence (1) and absence (0) for each sample and were transformed into a binary character matrix. Genetic distances were determined using GenALex 6.5 software.

Results

In *M. jankae*, short-term culture was efficiently made through multiple axillary shooting (Holobiuc et al. 2017, in press), minimal culture or slow-growth being the next level of *ex situ* preservation using *in vitro* methods. The behavior of the explants concerning the growth and regeneration depends on the factors modulated and the time interval recorded (Table 2). The use of minimal media with macro salts reduced at half (V2) or sucrose reduced at 10 g/l did not influence the growth or lateral shoots during two months. In the third month, only regeneration on V3 variant with reduced level of sucrose was lower. In the case of V1-V3 variants having similar growth rate to the control, the cultures could not be maintained more than 2-3 months. After two months of culture, the growth retardation effect was obtained applying different factors (Fig. 1 A-D). The use of ABA at the 37.8 µM level reduced the growth and lateral shooting was limited at 3-5 shoots/initial explant (Fig.1A). The application of clormerquat at both levels (1.26 and 2.5 mM) also determined a growth reduction, correlated with a good regeneration rate (Table 2, Fig. 1B), even after six months of culture (Fig. 1E). The origin of the new formed shoots are the lateral meristems of the double nodes stem explants.

Testing of several concentrations of flurprymidol showed that 32 to 64 µM levels induced significant growth retardation- (Table 2), this behavior being maintained over six months with an acceptable viability rate of the cultures (Table 3). The level of 48 µM flurprymidol allowed a good response of regeneration even after six months, 18 shoots/initial explants being recorded (Fig. 1F). Mannitol at 0.16 M reduced the growth and also allowed a positive rate of regeneration (Fig.1D, 1G).

Rooting process occurred easily on all media without retardation factors (V1-V3). In the case of growth-limitation factors, only 0.16 M mannitol sustained some roots formation beginning the first month, while at the 0.32 M level the rooting rate was recovered after three months (Table 3). Mannitol ensured rooting with acceptable rates (80%) just in the third month of preservation. ABA inhibited root

Fig 1. Growth retardation induced with different factors after 2 months of culture compared to the control **(**A - ABA 37.8 µM, B- clormerquat 1.26 mM, C- flurprymidol 48 µM, D - mannitol 0.16 M.); E. Details of medium-term regenerative cultures maintained during six months in the presence of clormerquat 2.5 mM, flurprymidol 48 µM (F), mannitol 0.16 M (G) (bar-1cm).

formation. In the presence of clormerquat, rooting was induced beginning the second month of culture. Flurprymidol application initial inhibited roots development. Rooting occurred with reduced rates only at 16 and 32 µM level after two months, roots developed slowly, but 64 µM inhibited rooting. To obtain well rooted plants, shoots were cultured further on medium without retardants.

The assessment of the genetic stability of regenerants from medium-term cultures

In vitro culture *per se* and the addition of different compounds can induce somaclonal variation consisted in different type of DNA alterations (Karp 1994). For this reason, for conservative purpose it is important to evaluate the genetic stability/variability of preserved plant material.

In our study, from seven tested ISSR primers, only four provided clear and scorable bands with satisfactory intensity for clone 1 and 3 primers for clone 2. The analysis of patterns amplification in clone 1 (Fig. 2 A-D) showed a total of 775 bands generated for 30 regenerants derived from medium-term cultures. The four tested primers generated 31 loci from which 10 polymorphic loci, the total percentage of polymorphism in this

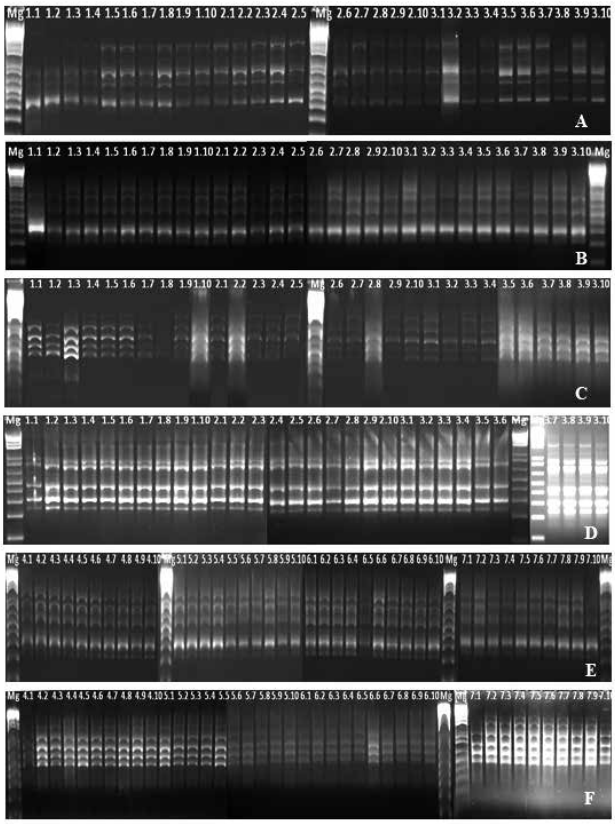

Fig. 2. ISSR amplification profiles of *M. jankae* regenerants belonging to clone **1** (1.1-1.10-control, 2.1-2.10- derived from variant 0.16 M mannitol, 3.1-3.10-derived from variant 37.8 µM ABA) using MC2 (A), MC3 (B), MC4 (C) and MC5 (D) primers and clone **2** (4.1-4.10- control, 5.1-5.10-derived from variant 2.5 mM clormerquat, 6.1-6.10- from 32µM flurprymidol, 7.1-7.10- from 48 µM flurprymidol) using the MC3 (E) and MC4 (F) primers.

clone was approximately 14%, mostly due to the 3 individuals in the control group that showed polymorphism by amplification with 2 from 4 primers. However, the number of samples that showed polymorphism is small (Table 1), and the genetic diversity among regenerants in the same experimental variant as well as per total for clone 1 have minimal values. In case of the second clone (Fig. 2 E, F), the amplification profiles for all three primers that produced reproducible bands, MC2, MC3 and MC4 showed the presence of 21 loci from which only 5 were polymorphic. The percentage of loci per clone is approximately 13% comparable to clone 1. Genetic diversity is very low for regenerants obtained from 2.5 mM clormerquat-supplemented medium and 32 µM flurprimydol treated regenerants. For regenerants obtained from cultures on higher level of flurprymidol (48 µM), genetic diversity was slightly increased than control (Table 4).

Discussion

Despite of the advantages of long-term preservation, minimal-slow growth is also considered to be useful for the conservation of plant crop genetic resources (Sarkar et al. 2001) and also for wild threatened plants (Reed et al. 2011). Among the factors tested to establish medium-term cultures in *M. jankae*, mannitol, is a sugar alcohol which was successfully used in medium-term cultures alone or in combination with other factors, at concentrations between 15-90 g/l (Hao & Deng 2003; Holobiuc et al. 2010, 2014). Usually, *in vitro* sugars and sugar alcohols provide the carbon source, but higher levels induce osmotic stress and decrease leaf water potential and also determine the growth reduction. In our experiment, mannitol induced a growth retardation lower than flurprymidol, but more than clormerquat, this effect being detected after two months. The regeneration through axillary shooting occurred at good rate in the presence of 0.16 M mannitol, in *M. jankae*, mannitol being tolerated at lower level than in related species *D. trifasciculatus* Kit ssp. *parviflorus* (Holobiuc et al. 2014).

For medium-term preservation, owing to the concern that osmotic compounds may affect the genetic stability through hypermethylation (Harding 1991) or because of abnormal physiological effects which can be inherited (Sarkar et al. 1999), growth retardants were also widely used (Sarkar et al. 2001). Abscisic acid (ABA) is a plant hormone (PGR) which besides its roles in leaves abscission, stress and plant pathogenesis, inhibits the cell division in the vascular cambium, stopping plant growth (Renau-Morata et al. 2006). The use of 37.8 µM ABA reduced the growth level, but few lateral shoots formed, without rooting. The survival rate was acceptable after seven months, while in *Dianthus* sp. ABA at similar level totally inhibited lateral meristems development from the explants (Holobiuc et al. 2010). Previously, ABA (0.1 mg/l) combined with ancymidol at 5 mg/l were also successfully used for minimal cultures induction in *Glycyrrhiza glabra* L. (Srivastava et al. 2013). Clormerquat usually is applied to promote flowering or inhibit ornamental plant elongation, being relatively safe and short-lasting (being metabolized). In our study, this compound had also a positive effect on growth limitation and promoted good regeneration rate through axillar shooting even after six months of maintenance. Flurprymidol reduces internode elongation through the inhibition of gibberellic acid (GA) biosynthesis. In *M. jankae*, flurprymidol at 35-48 µM levels proved to

Table 2 Parameters used to evaluate medium-term culture in *M. jankae* after different time intervals (values marked with the same letter are not significantly different at p≤0.05)

Variant	Maximum growth of the shoots (cm) / initial explants Interval of culture (months)				Number of shoots/ initial explant Interval of culture (months)			
	1	2	3	6	1	2	3	6
V1	6.02± 6.452a	9.82± 0.38 a	8.67± 0.54a	-	2.76±1.50a	4.8±2.17a	4.8±1.11a	-
V2	8.2± 6.56a	9.48± 0.25 a	10.2± 0.34a	-	2.84±1.62a	5.16±3.06a	6.72±2.76a	-
V3	5.32± 6.472a	7.084± 0.80 a	5± 0.76b	-	2.68±0.94a	3.4±1.19a	4.36±2.76a	-
V4	2.32 ± 0.452ab	2.016± 0.44b	2.07± 0.38cd	0.56± 0.18c	1.68± 0.47b	2±0.64b	1.96±1.62b	5.4±2.67a
V5	0.58± 0.82b	1.76± 0.42 bd	1.90± 0.41cd	0.60± 0.10c	1.64±0.48 b	1.92± 0.57b	2.16±0.68b	3±2.67c
V6	0.86± 1.044b	2.28± 0.16b	2.88± 0.27c	3.16± 0.50a	2.04±0.61a	2.12±0.72b	4.1±2.04a	6.25±1.68a
V7	1.62± 1.236b	2.37± 0.53b	2.33± 0.30c	2.06± 0.33b	1.72±0.97b	3.84±2.13a	5.68±2.42a	6.68±2.83a
V8	1.98± 1.652b	4.57± 1.25c	5± 0.55b	4.71± 0.89a	1.36±0.63b	1.84±1.14b	2.56±1.32b	3.08±1.07c
V9	0.56± 0.436b	0.75± 0.11d	0.98± 0.30d	0.55± 0.25c	1.36±0.48b	2.52±1.53a	4.24±2.35a	4.6±3.87b
V10	0.36± 0.532b	0.65± 0.16d	0.81± 0.19d	0.32± 0.14c	1.24±0.43b	1.6±0.5b	2.4±1.11b	7.08±4.99a
V11	0.42± 0.432b	0.58± 0.17d	0.66± 0.18d	0.52± 0.15c	1.4±0.57b	2.24±0.83b	2.56±1.11b	2.88±1.73c
V12	0.42± 0.452b	1.06± 0.28bd	1.11± 0.24cd	1.17± 0.30bc	1.8±0.57b	3.88±1.83a	5.68±3.59a	5.64±3.60a
V13	0.32± 0.348b	0.46± 0.11d	0.45± 0.15d	0.59± 0.13c	1.32±0.47b	2±0.64b	2.2±0.91b	3.96±3.52b

Table 3. Rooting and survival rate registered after different time intervals.

Variant	Composition	Rooting rate (%) interval of culture (months)			Survival rate (%) after 6 months
		1	2	3	6
V1	MS	100 a	100a	100a	-
V2	MS ½	100a	100 a	100a	-
V3	MS+ 10 g	100 a	100a	100 a	-
V4	MS+ABA 18.9 µM	0	0	0	92±0.12a
V5	MS+ABA 37.8 µM	0	0	0	88±0.12a
V6	MS+C 1.26 mM	0	64±0.28b	92±0.12a	96±0.09a
V7	MS+C 2.5 mM	0	28±2.47c	72±0.15b	96±0.09a
V8	MS+F 16 µM	0	36±2.01c	56±2.21c	100 a
V9	MS+F32 µM	0	36±2.02c	76±0.34b	88±0.12a
V10	MS+F48 µM	0	0	0	88±0.22a
V11	MS+F 64 µM	0	0	0	76±0.12ab
V12	MS+Man 0.16 M	32±1.96b	69±1.26b	81±1.33b	88±0.12a
V13	MS+Man 0.32 M	0	24±1.83c	80±0.18b	52±0.22b

Table 4 Genetic distances and genetic identity between regenerants from each experimental variants with growth retardants and control (clone 1 - 37.8 µM ABA(V5), and 0.16 M mannitol (V12), respectively clone 2 - 2.5 mM clormerquat(V7), 32 µM (V9) and 48 µM (V10) flurprymidol).

Experimental variants		Genetic distance	Genetic identity
Clone 1	**V12**	0,033	0,967
	V5	0,040	0,961
Clone 2	**V7**	0,015	0,985
	V9	0,015	0,985
	V10	0,087	0,917

be more efficient concerning growth limitation of shoots. The regeneration at 35-48 µM had a positive response concerning regeneration and survival even after six months of maintenance in restricted growth conditions.

For the genetic evaluation of regenerants derived from medium-term cultures, we used ISSR markers which are considered relevant for their genetic stability/variability assessment. Somaclonal variation can be easily and rapid detected using ISSR primers consisted in microsatellites sequences being a dominant marker (Butiuc-Keul 2006) ensuring a rapid evaluation of repetitive sequences susceptible to mutations. In a related taxon of *M. jankae*, *D. giganteus* D' Urv ssp. b*anaticus* (Heuff.) Tutin, Jarda et al. (2014) performing the genetic analysis of donor plants and regenerants concluded that ISSR markers detected higher polymorphism than SSR ones

In *M. jankae*, because in the case of the majority of the tested primers, polymorphic bands absented in the DNA pattern of regenerants compared to the control, a low genetic variability was detected. In other studies, based on similar analyses, the absence of genetic variations was also reported: in potato (Sarkar et al. 2001), in almond regenerants (Martin et al. 2004), in *Swertia chirayita* (Roxb. ex Fleming) H. Karst. (Joshi & Dhawan 2007). Soni & Kaur (2014) also recorded a very low level of polymorphism between *in vitro* regenerated clones in *Viola pilosa* Blume.

The evaluation of medium-term cultures in *M. jankae* as alternative preservation strategy after 7 months of maintenance in the presence of different factors with growth limitation effect, showed that clormerquat, flurprymidol and mannitol are suitable for minimal cultures, being compatible with good plant regeneration and cultures survival. The results of genetic assessment of regenerated clones in *M. jankae* proved that the methods of growth retardation using factors as ABA, clormerquat, flurprymidol and mannitol did not affect the genetic stability of regenerated plants from medium term culture.

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