Romanian Biotechnological Letters *Vol. 19, No. 5,2014*

ORIGINAL PAPER

Genetic stability of micropropagated *Iris germanica* L. varieties assessed by RAPD markers

Received for publication, January 20, 2014
Accepted, July 15, 2014

PETOLESCU CERASELA*, LAPADATESCU SIMONA*, VELICEVICI GIANCARLA*, DANCI MARCEL*, LAZAR ALEXANDRU*, CRETESCU IULIANA*, BALA MARIA*
**Banats University of Agricultural Sciencies and Veterinary Medicine, Timişoara, 300645, Romania*
E-mail address:cerapetolescu@yahoo.com

Abstract

The objective of this study was the improvement of in vitro plant regeneration of few German iris varieties (Blue Bird Wine, Tangerine Charm, Glazed Orange, Jane Philips) via somatic embryogenesis using leaf base. For callus and embryogenesis induction MS medium added with dichlorophenoxyacetic acid, α-nafthyl acetic acid, and kinetin was used. The frequency of explants with regeneration ability was up to 40% in all four varieties selected for this study. The number of regenerants per explant varied between 0 and 8.

For micropropagation, the most crucial aspect is to maintain the genetic conformity, the regenerants have to be identical to the mother plant. For the assessment of in vitro stability/ instability of the regenerated plants, we used RAPD molecular markers. For DNA extraction young leaves from 10 in vitro growing plants were sampled. The 10 RAPD primers were tested for detection of the genetic polymorphism among regenerated and mother plants. Only 5 produced reproducible fragments. RAPD analysis did not reveal any type of polymorphism between randomly selected in vitro plants and the mother plant, indicating the clonal nature of progenies. Our results showed an advantage of this in vitro propagation protocol because the risk of somaclonal variation is reduced.

Key words: genetic stability, German iris, RAPD markers, somatic embryogenesis

1. Introduction

Iris germanica is a species belonging to the genus *Iris* that includes over 300 species (fam. *Iridaceae, Monocotyledons*), spreaded mostly across the northern temperate zone (SCHULZE [1]). Most of European species, comprise rhizomatous irises with bearded outer tepals (Mathew [2]). In addition to their ornamental value, *I. germanica* contains isoflavones important for pharmaceutical purpose (ATTA-UR-RAHMAN et al. [3], TOMOYOSHI et al. [4]).

As other ornamental monocotyledonous species with bulbs or rhizomes, irises are generally vegetatively propagated but with a limited rate. The cross-pollination and extremely long seed germination of many species (from 2 to 5 years) makes the seed reproduction inefficient; moreover, it can additionally lead to splitting of the desired characters of plant (JEHAN et al. [5]). There are some studies regarding *in vitro* propagation in some *Iris* species: *I. setosa* (BOLTENKOV et al. [6]), *I. atrofusca* and *I. petrana (*AL-GABBIESH A et al. [7]*) I. siberica* (G. LAUBLIN [8]), *I. pallida* (GOZU et al. [9]), *I. germanica* (SHIMIZU et al. [10]). Plant regeneration from cell suspensions and protoplasts was achieved only in *Iris germanica* (WANG et al. [11]).

The decorative and biological values of *I. germanica*, justify to initiate their vegetative propagation through *in vitro* cell cultures. Tissue culture can be used as an alternative to conventional methods of vegetative propagation with the goal of enhancing the rate of multiplication of desired genotypes (PAEK and MURTHY [12]).

Molecular markers and molecular sequences contain useful information about evolutionary history (HAYMER [13]). The discriminative power of DNA markers as tool to characterize genetic diversity is very important because they can be used to assess the polymorphism of *in vitro* regenerated plants (KUMAR et al., [14]; MARTINS [15]).

The arbitrarily primed polymerase chain reaction (or RAPD) amplifies anonymous fragments of DNA from any genome (WILLIAMS [16]). RAPD-PCR are currently used as genetic markers quite useful in breeding programs for assessment of genetic variability between genera, species, populations, cultivars and very related lines (TAWAR [17]). The ability of RAPDs to produce multiple bands using a single primer means that a relatively small number of primers can be used to generate a very large number of amplicons from different regions of the genome. As consequence, multiple loci may be examined very quickly [OVESNA [18]).

For the assessment of genetic diversity of the regenerated plants belonging to some German iris varieties, compared with mother plants, we use RAPD markers. Several authors have used molecular markers to monitor genetic stability of *in vitro* culture-derived plants [TAWAR et al. [17]; MIÑANO et al [19]; GAGLIARDI et al, [20]; KOZYRENKO et al, [21]). For micropropagation, the most crucial aspect is to maintain the genetic conformity with the mother plants.

The objective of our study was to establish an efficient and reproducible plant regeneration protocol in few German iris varieties that would be suitable for mass propagation of this taxon with ornamental and/or pharmaceutical value. Using leaf base as explant, *in vitro* plant regeneration *via* somatic embryogenesis was induced. The results of the evaluation of genetic identity of the regenerants, using five RAPD primers, are also presented.

2. Materials and methods

Plant material

Induction of somatic embryogenesis

For *in vitro* induction of somatic embryogenesis leaves base explants were collected from *Iris germanica* plants grown in a greenhouse. Leaves removed from the plants were first thoroughly washed under tap water and immersed in 70% ethanol for 2 min, and then in 5% sodium hypochlorite with 0.1% Tween 20, for 10 min. After two rinses with sterile water, one or two leaves were removed under aseptic conditions (KEREŠA et al., [22])**.** Leaf bases (white parts of leaves proximate to the rhizome) were cut into segments and were once again sterilized by immersion in 70% ethanol for 10 sec., and then in 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 3 min, followed by four washes with sterile distilled water.

For callus and embryogenesis induction was used MS medium (MURASHIGE and SKOOG [23] , added with 4.52 μM with 2.4-dichlorophenoxyacetic acid (2.4-D), 4.83 μM α-naphthyl acetic acid (NAA) and 0.46 μM kinetin (Kin), solidified with agar. Callus induction media used in this experiment had the same plant growth regulators composition as the combination used in the work of KEREŠA et al., [22]). The media were adjusted to pH 5.8 before sterilization. All culture media were autoclaved at 121^0 C, 1 bar, for 30 min.

Five explants were cultured on each sterile Petri dish (90 mm diameter) containing 20 ml of medium sealed with Parafilm (five explants x five Petri dishes per each genotype). Cultures were incubated at 25 ± 1^0C under low light intensity and 16 h photoperiod. Calli were

maintained by sub-culturing on the same fresh callus induction medium as needed. Embryogenic calli were then subcultured on hormone-free medium containing MS salts and vitamins. Transfer of embryogenic calli to this medium enabled somatic embryos to maturate.

Three months after the beginning of the experiment, germinated somatic embryos were transferred to Erlenmayer flasks on hormone-free medium containing MS salts and vitamins, solidified with agar. Somatic embryos germination and transfer to hormone-free medium occured over about two months. Somatic embryos rooted abundantly and frequently produced new microshoots. The regenerants were maintained in aseptic conditions for further growth. Developed plants were transferred to pots and grown in growth chamber under a 16 h photoperiod. For DNA extraction young leaves from *in vitro* growing plants were sampled.

RAPD analysis

DNA extraction. Total genomic DNA of regenerated plants via somatic embryogenesis and from mother plants was extracted using Maxwell™ 16 Instrument from Promega. Purified concentrated products are obtained at high quality and high yield and can be used directly in a variety of downstream applications.

Primers and RAPD-PCR assay. The 5 primers were used for the detection of the genetic polymorphism among regenerated plants and mother plants: OPA09 (GGGTAACGCC), OPA14 (TCTGTGCTGG), OPA15 (TTCCGAACCC), OPA01 (CAGGCCCTTC), OPX02 (TTCCGCCACC) (SHIMITZU et al [10]; KOZYRENKO**,** [21])

The amplifications were carried out in a 25 µl PCR buffer containing: GoTaq® Green Master Mix ready-to-use solutions (GoTaq® DNA Polymerase, dNTP, $MgCl_2$, and reactions buffers at optimal concentrations for efficient amplification of DNA templates), RAPD primers, DNA template and Nuclease-Free Water. GoTaq® Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during eletrophoresis. DNA amplification was carried out using a Thermal-cycler by Corbett and reactions were submitted to the following PCR program: preliminary DNA denaturation for 5 min at 94°C, followed by 45 cycles consisting of denaturation (1 min, 94°C), primer annealing (1 min, 36°C), and extension (2 min, 72°C). A final extension for 3 min at 72°C was included. The RAPD products were separated by electrophoresis in 2% agarose gels, which run with 1xTAE buffer. Photos documentation was performed under UV light using a photo imaging system. Number of bands generated by different pairs of primer used was scored and the frequency of polymorphism was calculated.

Data analysis. Data were analyzed by current statistical methods. The amplification bands were scored as (1) and (0) on band (allele) presence and absence, respectively. The frequencies of the RAPD fragments were estimated for each of individuals from each genotype. Sizes amplification bands were estimated using PhotoCapt Molecular Weight System.

3. Results and Discussions

Somatic embryogenesis and plant regeneration

Leaf bases were cultured and compact yellow or white calli with rough surface, were obtained along their edges. Callus induction rate was 60%. After the first subculture, compact organogenic calli with green nodules were also formed.

The first embryos were developed at the surface of the embryogenic calli and were similar to the somatic embryos described by JEHAN et al. [5] with *I. pallida* and by RADOJEVIĆ and SUBOTIĆ [24] in *I. setosa*. Not all explants of the tested varieties produced embryogenic callus, moreover, some embryos failed to develop.

The same aspect was reported in plant regeneration of *I. setosa* (RADOJEVIĆ and SUBOTIĆ [24]).

Embryogenic calli were then subcultured on hormone-free medium containing MS salts and vitamins. Transfer of embryogenic calli to this medium enabled somatic embryos to maturate.

The frequency of mature somatic embryos was up to 20% in all four varieties selected for this study because some embryos failed to develop.

The number of regenerants per explant varied between 0 and 9 (Table 1). All tested genotypes had regeneration ability (Fig. 1). Larger clusters of mature somatic embryos were divided into smaller pieces in order to improved germination of somatic embryos and were placed on the same medium.

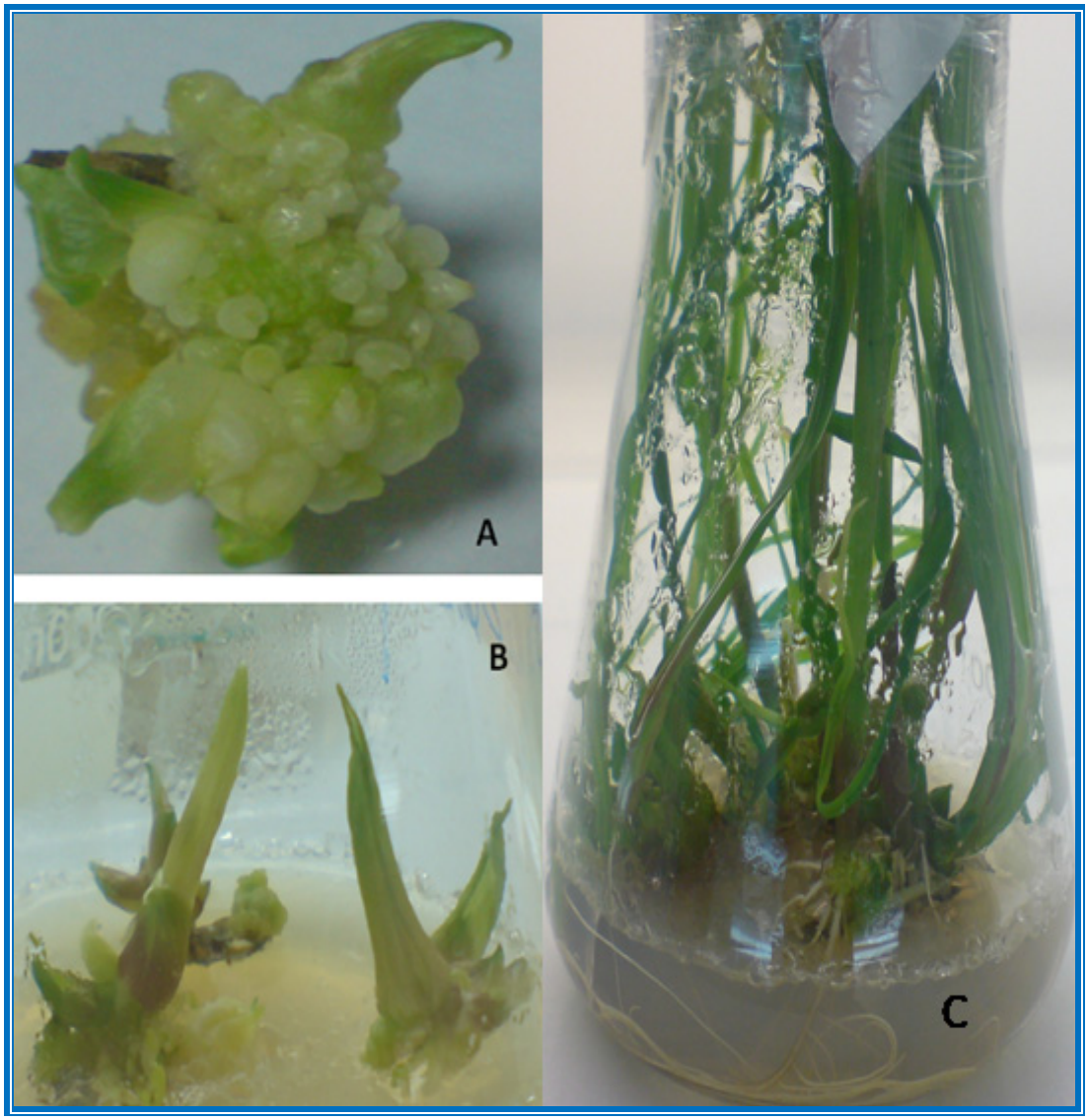

Figure 1. Jane Philips genotype: mature somatic embryos (A), regenerants (B, C)

Around 40-50 plantlets per genotype were produced through somatic embryogenesis. Plants were transferred to peat and grown in growth chamber for acclimatization.

Table 1

Number of regenerants per explant

No. Crt.	Genotype	Explants number	Number of regenerants/ explants $\bar{x} \pm s_{\bar{x}}$
1	Blue Bird Wine	25	**2,8±0,5**
2	Tangerine Charm	25	**2,9±0,5**
3	Glazed Orange	25	**2,6±0,5**
4	Jane Philips	25	**3,2±0,5**

Results are expressed as mean ± standard error (SE).

In our experiment, the frequency of mature somatic embryos obtained through callus culture induced from leaf base explants was higher than reported in *I. germanica* (4.0%) JEHAN et al. [5]. The best results were obtained in Jane Philips variety.

Several authors have reported different rates of regeneration in various *Iris* species, using embryogenesis or organogenesis **(**RADOJEVIĆ and SUBOTIĆ**,** [24]**;** SHIBLI and AJLOUNI, [25]; JEVREMOVIĆ and RADOJEVIĆ, [26]) or in suspension culture (SHIBLI and AJLOUNI, [25]; JEVREMOVIĆ and RADOJEVIĆ, [26]).

Somaclonal variation at molecular level

The 10 primers were screened for their ability to generate RAPD polymorphic DNA bands. Only 5 primers were selected for studying the genetic variability. The number of total bands is shown in table 2.

Table 2

RAPD primers used, number and size of bands detected for each genotype

Primer	Nucleotide sequence (5'→3')	Blue Bird Wine		Tangerine Charm		Glazed Orange		Jane Philips	
		Bands no	Size bands (pb)	Bands no	Size bands (pb)	Bands no	Size bands (pb)	Bands no	Size bands (pb)
OPA09	GGGTAACGCC	6	450-900	5	400-800	7	400-1400	7	450-1350
OPA14	TCTGTGCTGG	4	300-780	5	370-880	5	420-950	6	500-1100
OPA15	TTCCGAACCC	9	350-1350	8	300-1250	8	300-1150	7	400-1200
OPA01	CAGGCCCTTC	3	550-1000	4	580-1050	5	320-1130	3	430-950
OPX02	TTCCGCCACC	4	320-980	6	300-1000	3	480-1100	5	350-1320

The RAPD primers tested in this study generated monomorphic amplicones ranging in size from 450-1400 bp (OPA09), 300-1100 bp (OPA14), 350-1350 bp (OPA15), 320-1130 bp (OPA01), 320-1320 bp (OPX02). The number of bands for each primer ranged from 5-7 (OPA09), 4-6 (OPA14), 7-9 (OPA15), 3-5(OPA01), 3-6 (OPX02).

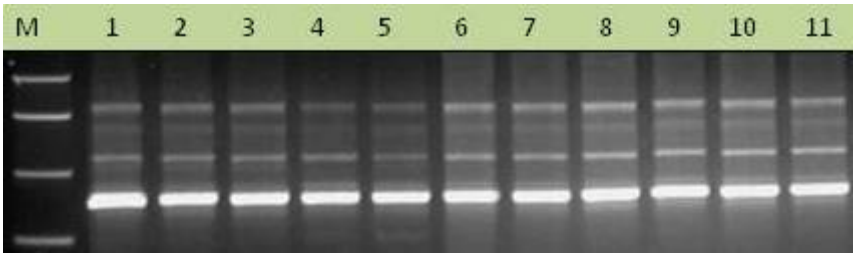

Fig.2. Analysis of 2% agarose gel electrophoresis for amplified products using OPA09 primer Tangerine Charm genotype – M - molecular marker; 1-10 regenerants; 11 explant donor.

An example of PCR amplification products obtained using the primer OPA09 of Tangerine Charm genotype is shown in figure 2.

In our study, RAPD analysis did not reveal any type of polymorphism between randomly selected *in vitro* plants and the mother plant, indicating the clonal nature of progeny. Similar results were obtained by BUBLYK [27] and PIÑA-ESCUTIA [28]. This results proved that the *in vitro* regeneration procedure used by us for the propagation of different varieties of *Iris germanica* is suitable because minimizes the risk of genetic variation.

4. Conclusions

In all four *Iris germanica* varieties selected for this study, the frequency of explants with regeneration ability was up to 20%.

The number of regenerants per explant varied between 0 and 8. Not all explants of the tested varieties produced embryogenic callus, moreover, some embryos did not converse into plants. The best results were obtained in Jane Philips variety.

This protocol represents an appropriate regeneration procedure for *I. germanica* varieties because no somaclonal variations were detected among the regenerated plants using RAPD analysis. OPA15 primer generated the largest number of bands, all monomorphic.

ACKNOWLEDGEMENTS

This work was published during the project "POSTDOCTORAL SCHOOL OF AGRICULTURE AND VETERINARY MEDICINE", POSDRU/89/1.5/S/62371, co-financed by the European Social Fund through the Sectorial Operational Programme for the Human Resources Development 2007-2013.

REFERENCES

1. W. SCHULZE, Wild-Ins für den Garten. - Bibliogr. Mitt. Universitätsbibl. Jena 48, Beih. zu: SCHULZE W. & VOGT H., Liliifloren. Internationale Auswahlbibliographie 1898-1986 (1988)
2. B. MATHEW, The Iris - B. T. Batsford Ltd., London, (1981)
3. ATTA-UR-RAHMAN, S NASIM, I BAIG, S JALIL, I ORHAN, B SENER, MI CHOUDHARY, Anti-inflammatory isoflavonoids from the rhizomes of Iris germanica, J Ethnopharmacol 86: 177–180 (2003).
4. A. TOMOYOSHI, I MASAYUKI, A. TOSHIO, A. SHIN-ICHI, Isoflavonoid production by adventitious-root cultures of *Iris germanica* (Iridaceae), Plant Biotechnology 22(3), 207–215 (2005)
5. JEHAN H, COURTOIS D, EHRET C, LERCH K, and PETIARD V. Plant regeneration of Iris pallida Lam. and Iris germanica L. via somatic embryogenesis from leaves, apices and young flowers. Plant Cell Reports 13: 671–675, (1994)
6. E.V. BOLTENKOV, L.N. MIRONOVA, E.V. ZAREMBO, Effect of phytohormones on plant regeneration in callus culture of Iris ensata Thunb. Biology Bulletin 34: 446–450 (2007).
7. A. AL-GABBIESH, D.S. HASSAWI, and F.U. AFIFI, In vitro propagation of endangered Iris species. Journal of Biological Sciences 6: 1035–1040 (2006).
8. G. LAUBLIN, HS SAINI, M. CAPPADOCIA, *In vitro* plant regeneration via somatic embryogenesis

from root culture of some rhizomatous irises. Plant Cell, Tissue and Organ Culture 27: 15–21 (1991).
9. Y. GOZU, M. YOKOYAMA, M. NAKAMURA, R. NAMBA, K. YOMOGIDA, M. YANAGI, S. NAKAMURA, In vitro propagation of Iris pallida. Plant Cell Reports 13: 12–16 (1993).
10. K SHIMIZU, H NAGAIKE, T YABUYA, and T ADACHI., Plant regeneration from suspension culture of *Iris germanica*. Plant Cell, Tissue and Organ Culture 50: 27–31. (1997)
11. WANG Y, JEKNIĆ Z, ERNST RC, and CHEN THH.. Efficient plant regeneration from suspension-cultured cells of tall bearded iris. HortScience 34: 730–735. (1999)
12. K.Y. PAEK and H.N. MURTHY, High frequency of bulblet regeneration from bulb scale sections of*Fritillaria thunbergii*. Plant Cell Tissue and Organ Culture, March vol. 68, no. 3, p. 247-252. (2002)
13. D.S. HAYMER, Random amplified polymorphic DNAs and microsatellites: What are they, and can they tell us anything we don't already know? Ann Entomol Soc Am 87, 717-22 (1994).
14. P. KUMAR, V.K. GUPTA, A.K. MISRA, D. R. MODI AND B. K. PANDEY**,** Potential of Molecular Markers in Plant Biotechnology**,** Plant Omics Journal, 2(4):141-162 (2009)
15. M. MARTINS, D.SARMENTO, M.M.OLIVEIRA, Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers, Plant Cell Reports, Issue 7, pp 492-496, (2004)
16. J.G. WILLIAMS, A.R. KUBELIK, K.J. LIVAK, J.A. RAFALSKI, TINGEY S.V. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers, Nucleic Acids Research 18: 6531-6535. (1990)
17. P.N. TAWAR, R.A. SAWANT DALVI, A. A. NIKAM, P. G. KAWAR, R.M. DEVARUMATH, An assessment of somaclonal variation in micropropagated plants of sugarcane by RAPD markers. Sugar Tech. 10: 124-127 (2008).
18. J. OVESNA, K. POLAKOVA, L. LEISOVA, DNA analyses and their application in plant breeding. Czech. J. Genet. Plant Breed.38:29-40 (2002)
19. H.S. MIÑANO, E. M. GONZÁLEZ-BENITO, C. MARTÍN, Molecular characterization and analysis of somaclonal variation in chrysanthemum cultivars using RAPD markers Scientia Horticulturae 122: 238-243 (2009)
20. R. F. GAGLIARDI, L. R. HANAI, G. PACHECO, C. A. OLIVEIRA, L. A. CARNEIRO, J. F MONTENEGRO VALLS, E MANSUR, M. L. CARNEIRO VIEIRA, Assessment of Genetic Stability Among *In Vitro* Plants of *Arachis retusa* Using RAPD and AFLP Markers for Germplasm Preservation. Journal of Integrative Plant Biology 49: **307 – 312 (**2007).
21. M. M. KOZYRENKO, E. V. ARTYUKOVA, L. S. LAUVE, AND E. V. BOLTENKOV, Genetic Variability of Callus Cultures of Some *Iris* Species, Biotechnologiya, , no. 4, pp. 38–48 (2002)
22. S. KEREŠA, A. MIHOVILOVIĆ, M. ĆURKOVIĆ-PERICA, B. MITIĆ, M. BARIĆ, I. VRŠEK, S. MARCHETTI, In Vitro Regeneration of the Croatian Endemic Species *Iris Adriatica* Trinajstić Ex Mitić, ACTA BIOLOGICA CRACOVIENSIA Series Botanica 51/2: 7–12 (2009)
23. MURASHIGE, T., F. SKOOG, 1962, A revised medium for rapid growth and bioassays with tobacco tissue culture, Physiol. Plant., 15, 43 1-497.
24. LJ. RADOJEVIC and S. SUBOTIC, Plant regeneration of *Iris setosa* Pall. Through somatic embryogenesis and organogenesis. J. Plant Physiol. 139:690-696 (1992)
25. SHIBLI RA, and AJLOUNI MM. Somatic embryogenesis in the endemic black iris. Plant Cell, Tissue and Organ Culture 61: 15–21 (2000)
26. S. JEVREMOVIĆ, and LJ. RADOJEVIĆ, Plant regeneration from suspension cultures of Iris pumila L., Acta Horticulturae 572: 59–65 (2002)
27. O. M BUBLYK, I. O. ANDREEV, K. V. SPIRIDONOVA, V. A. KUNAKH, Genetic variability in regenerated plants of *Ungernia victoris***.** Biologia Plantarum, (2011)
28. J. L. PIÑA-ESCUTIA, L. M. VÁZQUEZ-GARCÍA, A. M. ARZATE-FERNÁNDEZ**,** In vitro regeneration and genetic fidelity of Tigridia pavonia (L.f.) *DC***.,** Electronic Journal of Biotechnology, Vol.13 No.1, (2010)