African Journal of Biotechnology Vol. 10 (72), pp. 16181-16188, 16 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.807
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Somatic embryogenesis and bulblet regeneration in snakehead fritillary (*Fritillaria meleagris* L.)

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Accepted 27 December, 2010

Induction of *in vitro* morphogenesis of mature zygotic embryos of *Fritillaria meleagris* L. was investigated. Somatic embryogenesis and whole plant regeneration were achieved. Isolated zygotic embryos were cultured on MS medium that contained 3% sucrose, 0.7% agar, 250.0 mg/l casein hydrolysate, 250.0 mg/l L-proline, 80 mg/l adenine sulfate and 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) or thidiazuron (TDZ). Embryogenic callus was derived from mature zygotic embryos after 4 weeks on TDZ containing medium. Somatic embryos at the early stages of development arose from the surface of the embryogenic callus. Multiplication of somatic embryos, formation of bulblets and shoot development were observed on the same medium. Scale sections prepared from the formed bulblets were cultured on MS media supplemented with 2,4-D (0.1 to 10.0 mg/l) and TDZ (0.05 to 2.0 mg/l). Somatic embryos and bulblets were frequently produced from the scale sections. This is a successful report of plant regeneration through somatic embryogenesis for this very important medicinal and horticultural plant. Histological observation revealed that plants of *F. meleagris* L. were regenerated via somatic embryogenesis.

Key words: Bulbous plant, medicinal plant, snakehead fritillary, somatic embryos.

INTRODUCTION

Fritillaria species used as garden plants is well established and more recently it has become popular as cut flower for interior decorations and other floral arrangement. *Fritillaria meleagris* L., with the common name snakehead fritillary, is a well known horticultural plant belonging to the Lilliaceae family. Also, the bulbs of various species of the genus *Fritillaria* have been used as anti-tissuve and expectorant herbs used in traditional Chinese medicine for more than 2000 years (Li et al., 2000, 2001). Propagation of this plant through conventional methods via bulbs cuttings is limited due to a low survival rate. Bulb development is very slow (general 4 years of growth from initial seedling to maturity). Therefore, there is an urgent need to look for alternative means of propagation, which could ensure large-scale production of plants to fulfill the growing demands. *In vitro* propagation is a feasible alternative for the rapid multiplication and maintenance of germplasm. *In vitro* propagation ensures the production of true-to-type plants in limited time and space. The propagation from elite mature plants is preferred for this purpose as they are selected on past performances. Tissue culture studies in *Fritillaria* were initiated as early a 1977 (Sun et al., 1977). Since then, an increasing number of reports about *in vitro* regeneration protocols applied to the genus have been published for a number of *Fritillaria* species (Sun and Wang, 1991; Kukulezanka et al., 1989; Gao et al., 1999; Paek, 1994; 1996; Paek and Murthy, 2002; Özcan et al., 2007). Somatic embryogenesis is a multistep regeneration pathway starting with formation proembryogenic cell masses, followed by somatic embryo formation, germination and plant regeneration (Komamine et al., 2005). There are two paths of somatic embryogenesis; direct and indirect, and both can be undergone by many species (Quiroz-Figueroa et al., 2006). The use of plant growth regulators is useful in most species. 2,4-dichlorophenoxyacetic acid (2,4-D) such as auxin and thidiazuron (TDZ) such as

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cytokinin are especially significant in promoting somatic embryogenesis (Jiménez, 2005). Sometimes somatic embryos are recognized only by their external shape and morphology, although histological study is necessary to confirm the identification of different developmental phases (Yeung, 1999). Somatic embryogenesis has already been used as a method for conserving and safe-guarding overexploited and endangered plant species (Nadeem, 2000). Making use of *in vitro* culture can provide enough plant material to reinforce wild populations when appropriate. The choice of explants for establishment of *in vitro* culture is largely dictated by the method to be adopted for *in vitro* propagation. The ability of mature zygotic embryos to produce somatic embryos on induction medium clearly indicates that zygotic embryos are suitable materials for *in vitro* regeneration of *Fritillaria* species. Previous literature showed *in vitro* plant regeneration from bulb scales of *Fritillaria* species. The results from this study can be compared with commonly used bulbs as explants for *in vitro* regeneration. This is important for several reasons: bulbs are usually contaminated by pathogens; they have limited number scales and the use of bulbs could result in the destruction of *Fritillaria* natural populations (Witomska and Lukazewska, 1997). Induction of somatic embryogenesis in *F. meleagris* L. has not yet been reported. The first report of the induction of somatic embryogenesis in the genus was for *F. imperialis* (Mohammadani-Dehcheshmeh et al., 2007). The authors reported the induction of indirect somatic embryogenesis from the petal explants.

In this study, the induction of somatic embryogenesis and plant regeneration in callus cultures derived from mature zygotic embryos of *F. meleagris* L was investigated. Also, the effect of growth regulators and light condition on somatic embryos and bulblets formation from bulb scales was investigated. Up till now, no precise histological analysis of the different stages of development has been performed during the *in vitro* morphogenesis of *F. meleagris* L. A histological view of the developmental stages of somatic embryogenesis of *F. meleagris* L is presented in this study.

MATERIALS AND METHODS

Embryogenic callus induction from mature zygotic embryos

Seeds of *F. meleagris* L. were washed with running water for 1 h and then surface sterilized in 20% bleach solution (sodium hypochlorite) for 20 min. After sterilization, seeds were washed with sterile deionised water 3 times for 5 min. Mature embryos were isolated aseptically and were used as explants for embryogenic callus induction. All media were adjusted to pH 5.8 with 1 N NaOH and autoclaved at 121°C with 1.4 kg/cm^2 for 25 min. All cultures were maintained at 24 ± 2°C under fluorescent light of 40 µmol m^{-2}s^{-1} 16 h light / 8 h dark photoperiod. The basal medium (BM) consisted of MS medium (Murashige and Skoog, 1962), 30 g/l sucrose, 250 mg/l casein hydrolysate, 250 mg/l L-proline, 80 mg/l adenine sulfate and was solidified with 7 g/l agar. Isolated mature zygotic embryos were cultured 4 weeks on three induction media: hormones free BM, BM with 2,4-D 1.0 mg/ l and BM with TDZ 1.0 mg/l. Formed callus were cultivated on same media. Bulblet formation was observed on BM supplemented with TDZ 1.0 mg/l.

Somatic embryos and bulblets formation from bulblet scale sections

Bulbs formed on BM supplemented with TDZ 1.0 mg/l were cut on 4 scale sections and cultured on BM that contained different concentrations of 2,4-D in mg/l (0.1, 0.5, 1.0, 2.0, 5.0 10.0) and TDZ (0.05, 0.1, 0.2, 0.5, 1.0 and 2.0). Hormone free medium (BM) was used as control. Cultures were incubated for 4 weeks on light and dark conditions at 24 ± 2°C. After 4 weeks on induction media, the number of somatic embryos was measured per the number of embyogenic callus. Each experiment consisted of 10 explants per culture vessel and three replicate vessels per treatment. The experiments were repeated at least three times. Statistical analysis was performed using StatGrafics software version 4.2 (STSC Inc., Rockville, Marylend, USA). Data were subjected to analysis of variance (ANOVA) and comparisons between the mean values of treatments were made by the least significant difference (LSD) test calculated at the confidence level of $P \leq 0.05$. For bulblet formation, 4 weeks after initiation of culture, percentage of bulblet formation was measured by dividing the number of explants producing bulblets per number of cultured explants.

Rooting and acclimation

Regenerated bulbs were cultured on BM without growth regulators for 9 weeks at 4°C. The cultures were incubated for the same time on light conditions at 24 ± 2°C and were used as control. Rooted bulbs were transferred into soil, covered with glasses and moved to the greenhouse. This experiment involved 80 to 100 bulbs with three replications. After 2 weeks in the growth chamber, the regenerants were gradually exposed to reduced relative humidity by progressively removing the glass cover over a period of three weeks. Plantlets survived the acclimatization and grew slow and well.

Histological study

Ontogeny of somatic embryo development from mature zygotic embryos was studied histologycally. Embryogenic callus and somatic embryos were fixed in a solution of formalin, alcohol and acetic acid (FAA), 100 ml of which contains 5.4 ml formalin (37 %), 65.5 ml ethanol (96%), 5 ml glacial acetic acid and 24 ml distilled water (Jensen, 1962). Parts of the explants with different developmental structures were cut and embedded in Histowax (Sweden). At the beginning of this process, the samples were dehydrated in 2-h steps through a graded series of ethanol (50, 70, 96 and 100%). The samples were then embedded in Histowax (Histolab, Sweden) for 72 h at 58°C. Slices (5 µm) were cut at room temperature using rotary microtome (Reichert-Vienna) equipped with type 819 microtome blades (Leica, Germany). Slices were stretched on a drop of distilled water and mounted on slides. They were stained with haematoxyline (Jensen 1962). Sections were mounted in DPX before microscopic examination (Leica, Leitz DMRB, Germany).

RESULTS

Embryogenic callus induction from mature zygotic embryos

The BM containing 1 mgl^{-1} TDZ and hormone free medium

were the most efficient for inducing yellow-white and compact embryogenic callus (Figure 1 a). Embryogenic callus produced embryos on the induction medium itself (Figure 1 b). Embryogenic callus with embryos were used for further maturation experiments. Multiplication of somatic embryos and bulblet formation of *F. meleagris* L. were also achieved on BM containing TDZ 1 mgl^{-1} (Figure 1c and d). In the investigation, 2,4-D had no effects on embryogenic callus formation as well as somatic embryo and bulbs development (data not shown).

Somatic embryos and bulblets formation from bulblet sections

In this work, regeneration from *in vitro* bulblet sections of *F. meleagris* L was also investigated. Formation of somatic embryos and bulblets from sections was analyzed under continuous dark and under 16 h light / 8 h dark cycle at 24 ± 2°C. In the case of *F. meleagris* L., bulblet sections responded well to 16 h light / 8 h dark growth conditions when compared to continuous dark regime and also produced more somatic embryos bulblets. The BM medium without growth regulators induced a few somatic embryos and about two bulblets per explant. The number of developed somatic embryos was higher in cultures grown on BM supplemented with TDZ (Table 1, Figure 1 e, f and g) than on BM with 2,4-D on both treatments (light/dark). The highest number of somatic embryos was noticed on BM supplemented with TDZ (0.2 mgl^{-1}) under light condition and with TDZ (0.1 mgl^{-1}) under dark condition. The number of somatic embryos was concentration dependent and increased until the concentration of TDZ was 0.2 and 0.1 mgl^{-1} under light/dark, respectively. The number of formed somatic embryos constantly decreased on higher concentrations of TDZ. The highest number of somatic embryos on BM with 2,4-D was observed in 1 mg l^{-1} under light and dark conditions (Table 2). The number of somatic embryos was also concentration dependent and decreased on higher concentrations of 2,4-D. Generally, the number of formed bulblets was significantly smaller than the number of formed somatic embryos on the same media. The BM supplemented with TDZ was superior in the induction of bulblets under light (14.93%) and dark (3.6%) conditions. The maximum number (2.76) of bulblets was observed on BM supplemented with TDZ (0.05 mgl^{-1}) under light condition and maximum number (2.1) of bulblets under darkness condition was observed on BM with TDZ (0.5 mgl^{-1}). The number of bulblets formed on BM supplemented with 2,4-D was small under light condition (4.12%) as under darkness condition (2.32%).

Acclimation

The results indicated that cold treatment was suitable for overcoming the dormancy and rooting of the harvested bulblets and with this treatment, 60.45% of bulblets sprouted in *ex vitro* transplantation. The average number of roots per bulb was 2 and the average length of root was 8.49 mm (Figures 1h and i). Rooted plantlets from each treatment were acclimatized in greenhouse conditions (Figure 1j).

Histological analysis of somatic embryos development

After three weeks of somatic embryogenesis induction on BM medium with TDZ 1 mg/l, a proliferate burst in the epidermal and subepidermal layers and the beginning of cellular segregation was seen (Figure 2a). The intensive periclinal division of these cells led to the formation of distinct cell groups which could be interpreted as early stages in embryogenesis. These were present in groups of 6 to 8 isodiametric meristematic cells with prominent nuclei and dense cytoplasm (Figure 2b). Cell divisions then progressively became asynchronous and lost periclinal orientation, thus produced compact, smooth-surfaced and meristematic masses clearly delimited by a protoderm (Figure 2c). Such nodular structures were also formed deeply within the bulb or primary explant (Figure 2d). Further development of these proembryo structures led to the formation of globular somatic embryo at the surface of explant as shown in Figure 2e. All developmental stages on the explant in the same time indicated asynchronous development of somatic embryos. Well-organized globular shaped structures developed further through the characteristic heart-shaped stage to form a cotyledonary stage embryo (Figures 2f and g). The embryo had a shoot primordial enclosed within a pair of cotyledons and a distinct root primordium (Figure 2g). Moreover, this advanced stage or developed somatic embryos showed a contained provascular strand. The developing somatic embryos had no detectable vascular connection with primary explants. The cotyledonary node region programmed for shoot organogenesis showed development of a shoot primordium with a well-defined dome shaped apical meristem (Figure 2h).

DISCUSSION

The bulbous plants reproduced vegetatively and the process of multiplication was very low. Beside the advantages of efficient propagation, somatic embryogenesis has several basic applications to biotechnology of this plant including *F. meleagris* L. In this study, morphogenic responses obtained from mature zygotic embryos and bulblet scale explants of *F.meleagris* L. of known ornamental and medicinal value are of interest considering the potential of tissue culture for the improvement of plant conservation methods. Somatic

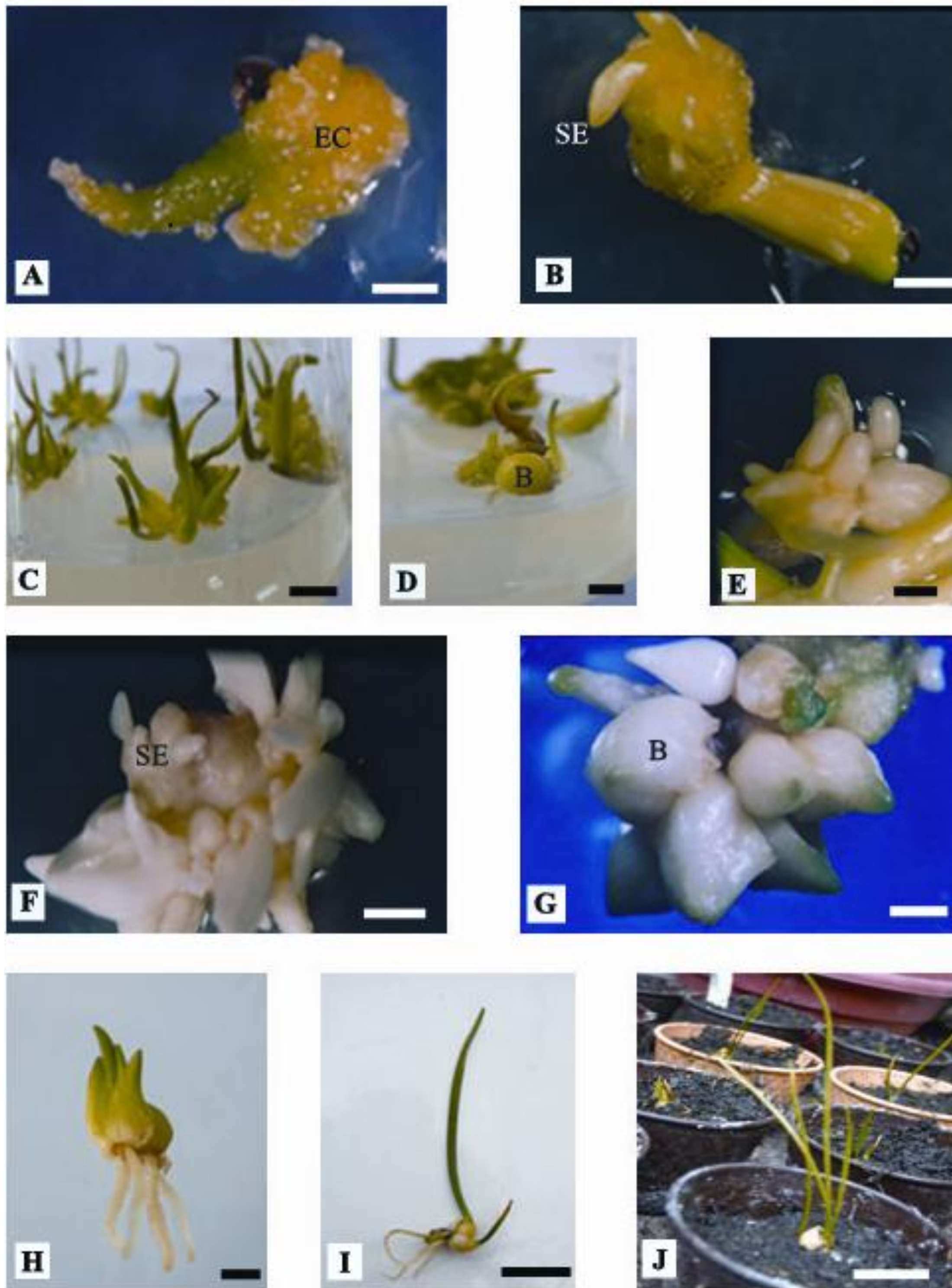

Figure 1. Differentiation of somatic embryos and bulblets of *F.meleagris* L. (A) Embryogenic callus induced on the BM containing 1.0 mgl^{-1} TDZ from mature zygotic embryos (bar = 0.5 mm); B, formation of somatic embryos on embryogenic callus (bar = 0.5 mm); C and D, somatic embryos and bulblets multiplication on medium with 1.0 TDZ mgl^{-1} (bar = 0.8 mm); E, Somatic embryos on bulb section on BM with: 0.1 mgl^{-1} TDZ under light condition (bar = 0.5 mm); F; with 0.1 mg l^{-1} TDZ under dark condition (bar = 0.5 mm); G, bulblets on bulb section on BM with 0.5 mgl^{-1} TDZ under dark condition (bar = 0.5 mm); H rooted bulb after 9 weeks cold treatment (bar = 1 cm); I, rooted plant on BM medium without hormone (bar = 1 cm); J, potted *in vitro* plantlets in greenhouse condition (bar = 1 cm); EC, embryogenic callus; SE, somatic embryo; B, bulblet.

Table 1. The effect of different concentrations of 2,4-D (mg l^{-1})on somatic embryos induction for *in vitro* bulblet sections of *F. meleagris* L.

Growth condition	Concentration of 2,4-D (mg l^{-1})						
	0	0.1	0.5	1.0	2.0	5.0	10.0
Light	$4.95±0.65^{a}$	$5.07±0.53^{bc}$	$4.66±0.43^{bc}$	$5.36±0.66^{c}$	$3.77±0.37^{ab}$	$2.46±0.25^{a}$	$2.38±0.28^{a}$
Darkness	$4.62±0.59^{a}$	$3.95±0.39^{b}$	$3.8±0.31^{b}$	$4.21±0.44^{b}$	$2.77±0.22^{a}$	$3.54±0.27^{ab}$	$3.32±0.37^{ab}$

Values shown are means of 3 replicates of 32 explants each. Values represent mean ± SE. Means followed by the same letters within columns are not significantly different according to LSD test at p ≤ 0.05 probability level.

Table 2. The effect of different concentration of TDZ (mg l^{-1}) on somatic embryos induction for *in vitro* bulblet sections of *F. meleagris* L.

Growth condition	Concentration of TDZ (mg l^{-1})						
	0	0.05	0.1	0.2	0.5	1.0	2.0
Light	$4.95±0.65^{a}$	$5.96±0.64^{b}$	$6.07±0.62^{b}$	$7.31±0.95^{b}$	$6.33±0.73^{b}$	$4.11±0.36^{a}$	$4.01±0.35^{a}$
Darkness	$4.62±0.59^{a}$	$3.0±0.31^{a}$	$6.55±0.78^{c}$	$6.11±0.57^{c}$	$5.44±0.57^{bc}$	$4.22±0.37^{ab}$	$3.20±0.31^{a}$

Values shown are means of 3 replicates of 32 explants each. Values represent mean ±SE. Means followed by the same letters within columns are not significantly different according to LSD test at p ≤ 0.05 probability level.

embryogenesis represents a simple and very efficient alternative means of regenerating large number of monocotyledonous geophytes such as saffron (Ahuja et al., 1994), *Narcissus* (Sage et al., 2000) and *Lilium* (Kim et al., 2003).

Explant source is one of the most important factors in the induction of morphogenetic response of *in vitro* cultures, especially in monocots where cells diffentiate early and quickly and so lose their morphogenetic potential (Krishnaraj and Vasil, 1995). Only parts of the plant that are close to meristematic tissue *in vivo* can respond to *in vitro* treatments. Zygotic embryos are frequently used as explants for initiating embryogenic culture and physiological conditions of the explants play an important role in the induction process. These highly regenerative organs can be an alternative source for *in vitro* propagation of rare species such as *F. meleagris* L. The type and concentration of plant growth regulators in the culture medium play an important role in induction and development of somatic embryos. The induction of somatic embryogenesis using only 2,4-D as a growth regulator has been observed in many monocotyledons species such as *Allium aflatunense* (Subotić et al., 2006), *Allium sativum* (Luciani et al., 2006) and rice (Meneses et al., 2005). Cytokinins are known to enhance plant differentiation and are mostly used in the regeneration medium in plant tissue culture. Thidiazuron is able to induce diverse morphogenic responses, ranging from tissue proliferation to adventitious shoot and somatic embryo formation. Of all the plant growth regulators used, embryo axes of *Allium magnum* cultured on the media that contained only TDZ (1.0 to 2.0 mgl^{-1}) and IAA (0.25 to 2.0 mg l^{-1}) produced green-yellowish and friable embryogenic calli (Xie and Hong, 2001). Apart from its cytokinin-like activity, TDZ has been suggested to be a modulator of the endogenous auxin level. Of interest in this contest is the modulation of endogenous auxin by TDZ (Hutchinson et al., 1996), since the induction of somatic embryogenesis is commonly associated with auxins (Visser et al., 1992). Previous *in vitro* culture studies of *Fritillaria thunbergii* showed that addition of growth regulators to the medium enhanced the effect on bulblet regeneration (Sun et al., 1977; Seon et al., 1999). In *F. melegris* L., typically bulbous plant somatic embryos led to formation of bulblets. Similar morphogenic pathways using petal explants was reported previously for *F. imperialis* (Mohammadi-Dehceshmen et al., 2007).

Bulblet scale explants are the most commonly used primary explants for *in vitro* propagation of *Fritillaria* species (Peak, 1996; Witomska, 2000). The results of usage of different concentrations of 2,4-D and TDZ in culture of bulblet scales of *F. meleagris*, did not only induce somatic embryogenesis, but also stimulated the formation of bulblet. Bulblets formation differed among the hormone concentrations in BM medium. The results indicated that a range of 0.05 to 0.5 mg l^{-1} TDZ was an optimal concentration for bulblets formation of *F. melegris* L. This was low when compared with the efficiencies reported for production of bulblets in culture of bulblets scale of *F. thunbergii* (Peak and Murthy, 2002). These authors established high frequency bulblets regeneration using various concentrations of cytokinis and NAA. To improve on the present results, many combinations of 2,4-D and TDZ in BM medium will be tested.

Light suppresses bulblet formation (Stimart and Ascher, 1978) and the dark was more favorable for bulblet regeneration in *Lillium* (Niimi and Onozawa, 1979). A similar effect of light/dark regimes was noticed during the

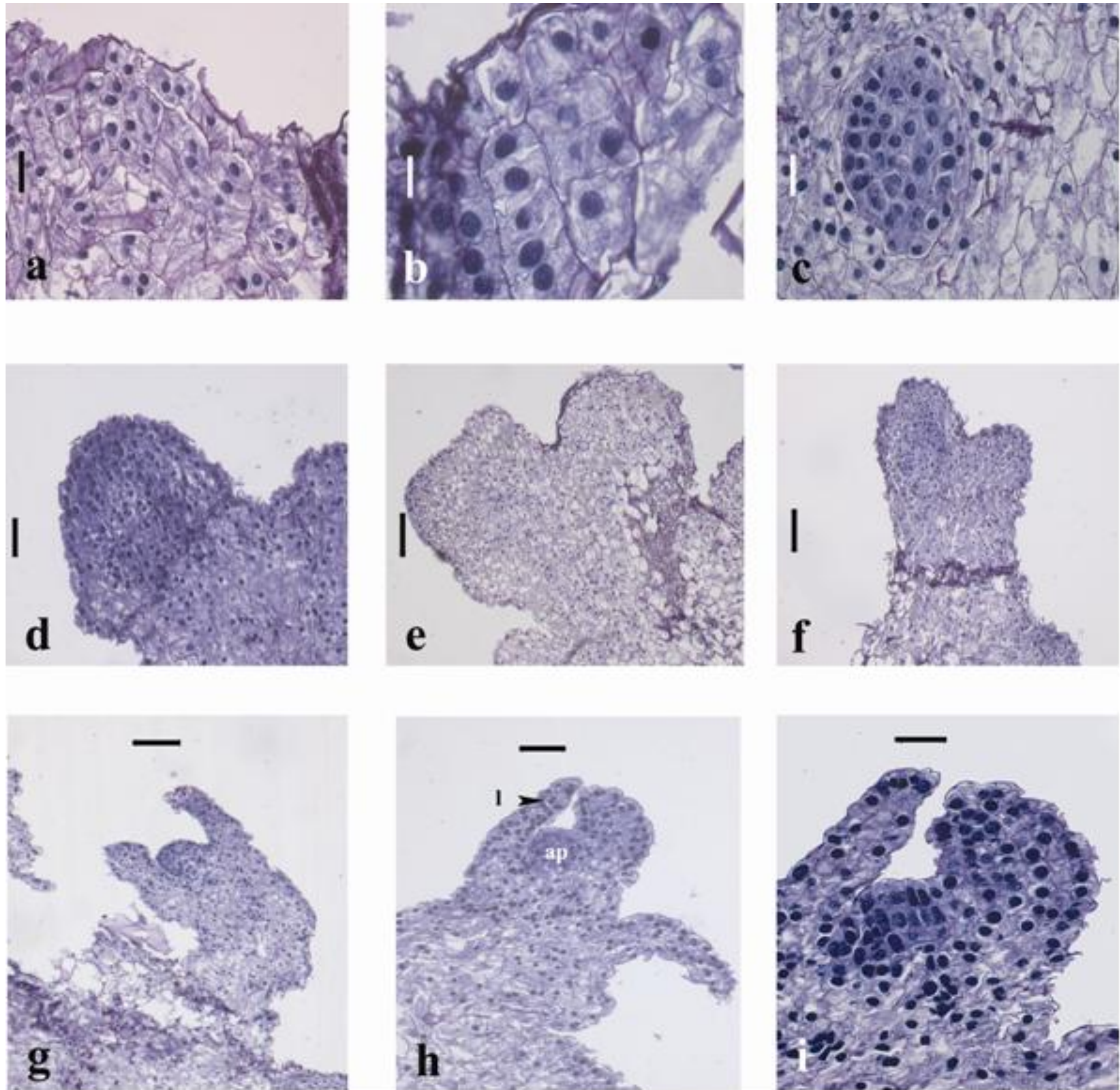

Figure 2. Histological observation of somatic embryogenesis from bulbs culture of *F. meleagris* L. (a) Cross-section of bulb showing small clumps of densely stained cells in subepidermal layer (bar = 60 µm); (b) Initial periclinal divisions in superficial layers of explant (bar = 30 µm); (c) Section showing extrusion through the epidermis of proliferating meristematic masses (bar = 60 µm); (d) Proembryogenic stages containing approximately 40 cells (bar = 120 µm); (e) Somatic embryos at the globular stages (bar = 240 µm); (f) Heart-shaped embryo with procambial strands (bar = 240 µm); (g) Cotiledonary embryo with a cotyledons, procambial strands and root meristem (bar = 240 µm); (h) Longitudinal section of somatic embryos with well developed apical meristem and leaf (bar = 120 µm); (i) Detail of apical meristem and well developed leaf (bar = 60 µm).

growth of bulblet of *Lillium longiflorum* (Leshem et al., 1982). In *Hyacintus*, effect of light or darkness on bulblet regeneration from flower buds was cultivar dependent (Kim et al., 1981). Light accelerated bulblet formation on all types of *Fritillaria imperialis* explant used (Witomska and Lukaszewska, 1997). Tissue cultures of fritillaries also need low temperature; callus and bulblets are normally induced at standard growth room temperature but in order to develop further growth of *in vitro* formed bulblets, they need 2 to 15°C of cold treatment (Peak, 1996). Cold treatment (4°C) had great positive effect on breaking the dormancy that resulted in the increase in rooting and sprouting of *F. meleagris* bulblets formed *in vitro*.

Structural analysis is an important step in the study of *in vitro* somatic embryogenesis (Yeung, 1999). The histology observations suggest that the first stage of development of somatic embryos was characterized by breaking

up of the meristematic masses. Active cell division in the superficial layers led to the formation of clusters that were composed of small isodiametric cells followed by the formation of epidermis. The stages of differentiation during somatic embryogenesis in *F. melegris* L. reported in this work are in agreement with observation previously reported in several geophytes as in the case of *Narcissus* (Sage et al., 2000) and *Allium sativum* (Fereol et al., 2000).

Conclusion

In vitro culture techniques are an important aid for the multiplication of plant species, which have limitations of conventional propagation. In this study, a protocol for somatic embryogenesis and bulblets regeneration of *F. meleagris* L. is envisaged as a means for germplasm conservation to ensure the survival of this endangered plant. This is an important step in for further studies of efficient regeneration from other sources of explants as well as genetic transformation in *F. meleagris* L.

ACKNOWLEDGEMENT

This research was financially supported by Serbian Ministry of Science and Technological Development-Project (No ON173015).

REFERENCES

Ahuja A, Koul A, Ram G, Kaul Bl (1994) Somatic embryogenesis and plant regeneration in saffron , *Crocus sativus* L. Indian J. Exp. Biol. 32: 135-140.

Fereol L, Chovelon V, Causse S, Michaux-Ferriere N, Kahane R (2002) Evidence of a somatic embryogenesis process for the plant regeneration in garlic (*Allium sativum* L.). Plant Cell Rep. 21: 197-203.

Gao SL, Zhu DN, Cai ZH, Jiang Y and Xu DR (1999). Organ culture of a precious Chinese medicinal plant-*Fritillaria unibracteata*. Plant Cell Tissue Org. Cult. 59: 197-201.

Hutchinson MJ, Murch SJ, Saxena PK (1996). Morphoregulatory role of TDZ: evidence of the involvement of endogenous auxin in TDZ-induced somatic embryogenesis of geranium (*Pelargonium × hortorum* Bailey). J. Plant Phys. 149: 573-579.

Jensen WA (1962). Botanical Histochemistry, Gauthier-Villars, San Francisco, CA, USA, 1-408.

Jiménez VM (2005). Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis. Plant Growth Regul. 47: 91-110.

Kim SK, Lee JS, Huang KH, Ahn BJ (2003). Utilization of embryogenesis cell cultures for the mass production of bulblets in Oriental and Easter Lilias. Acta Hortic. 625: 253-259.

Kim YJ, Hasegawa PM and Bressan RA (1981). *In vitro* propagation of *Hyacinthus*. Hort. Sci. 16: 645-647.

Komamine A, Murata N, Nomura K (2005). Mechanisms of somatic embryogenesis in carrot suspension cultures-morphology, physiology, biochemistry and molecular biology. *In vitro* Cell. Dev. Biol. Plant, 41: 6-10.

Krishnaraj S, Vasil IK (1995). Somatic embrogenesis in herbaceous monocots. In: Thorpe TA (ed) *In Vitro* Embryogenesis in Plants, Kluwer Academic Publishers, Dordrecht, pp. 417-470.

Kukulezanka K, Kromor K, Czastka B (1989). Propagation of *Fritillaria meleagris* L. through tissue culture. Acta Hortic. 251: 147-153.

Leshem B, Lilien-Kipnis H, Steintiz B (1982). The effect of light and of explant orientation on the regeneration and subsequent growth of bulblets of *Lillium longiflorum* Thumb. Bulb scale sections cultured *in vitro*. Sci. Hortic. 17: 129-136.

Li SL, Li P, Lin G, Chan SW, Ho YP (2000). Simultaneous determination of seven major isosteroidal alkaloids in bulb of *Fritillaria* by gas chromatography. J. Chromatogr. A 873: 221-228.

Li SL, Lin G, Chan SW and Li P (2001). Determination of the major isosteroidal in bulbs of *Fritillaria* by high-performance liquid chromatography coupled with evaporative light scattering detection. J. Chromatogr. A 909: 207-214.

Luciani GF, Mary AK, Pellegrini C and Curvetto NR (2006). Effects of explants and growth regulators in garlic callus formation and plant regeneration. Plant Cell Tissue Org. Cult. 87: 139-143.

Meneses A, Flores D, Munoz M, Arrieta G and Espinoza AM (2005). Effect of 2, 4-D hydric and light on indica rice (*Oryza sativa*) somatic embryogenesis. Rev. Biol. Trop. 53: 3-4.

Mohammadi-Dehcheshmeh M, Khalighi A, Naderi R, Ebrahimie E and Sardari M (2007). Indirect somatic embryogenesis from petal explant of endangered wild population of *Fritillaria imperialis*. Pak J. Biol. Sci. 10: 1875-1879.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Phys. Plant. 15: 473-497.

Nadeem M, Palni LMS, Purohit AN, Pandey, H, Nandi SK (2000) Propagation and conservation of *Podophyllum hexandrum* Royle: an important medicinal herb. Biol. Conserv. 92: 121-129.

Niimi Y, Onozawa T (1979). *In vitro* bulblet formation from leaf segments of lilies, especially *Lillium rubellum* Baker. Sci. Hortic. 11: 379-389.

Özcan S, Parmaksiz I, Mirici S, Çöçü S, Uranbey S, Ipek A, Sancak C, Sarihan E, Gürbüz B, Sevimay CS, Arslan N (2007). Efficient *in vitro* bulblet regeneration from immature embryos of endemic and endangered geophyte species in *Sternbergia, Muscari* and *Fritillaria* Genera. In: Xu Z, Li J, Xue Y, Yang W (Eds) Biotechnology and Sustainable Agriculture 2006 and Beyond, Springer, Berlin, Germany, pp. 381-383.

Paek KY (1994). Micropropagation of *Fritillaria thunbergii*. Research report of Ministry of Science and Technology in Korea, pp. 1-123.

Paek KY (1996). Several factors affecting bulblet regeneration from the culture of scale segment on none-bud in fritillary as medicinal bulbous plant. Acta Hortc. 440: 498-503.

Paek KY, Murthy HN (2002). High frequency of bulblet regeneration bulb scale sections of *Fritillaria thunbergii*. Plant Cell Tissue Org. Cult. 68: 247-252.

Quiroz-Figueroa F, Rojas-Herrera R, Galaz-Avalos RM, Layola,Vargas V (2006). Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. Plant Cell Tissue Org. Cult. 86: 285-301.

Radojević Lj, Subotić A (1992). Plant regeneration of *Iris setosa* Pall. through somatic embryogenesis and organogenesis. J. Plant Physiol. 139: 690-696.

Sage OD, Lynn J, Hammatt N (2000). Somatic embryogenesis in *Narcissus pseudonarcissus* cvs. Golden Harvest and St. Keverne. Plant Sci. 150: 209-216.

Seon JH, Paek KY, Gao WY, Park CH, Sung NS (1999). Factors affecting micropropagation of pathogen – free stocks in *Fritillaria thunbergii*. Acta
Hortic. 502: 333-337.

Stimart DP, Ascher PD (1978). Tissue culture of bulb scale section for sexual propagation of *Lillium longiflorum* Thumb. J. Am. Soc. Hort. Sci. 502: 180-184.

Sun CS, Wang DY (1991). *Fritillaria* spp. (Fritillary): *In vitro* Culture and Regeneration of Plants. Biotechnology in Agriculture and Forestry, Vol. 15 Medicinal and Aromatic plants III (eds.) Bajaj YPS, Springer-Verlag Berlin Hiedelberg, pp. 258-269.

Sun CS Chu CC, Wang DY (1977). Callus formation and organ formation in the tissue culture of *Fritillaria thunbergii* Miq. Acta Bot.
Sin. 19: 161-162. (in Chinese with English summary).

Visser C, Quereshi JA, Gill R, Saxena PK (1992). Morphoregulatory role of thidiazuron: substitution of auxin and cytokinin requirement for the

induction of somatic embryogenesis in geranium hypocotyl cultures. Plant Physiol. 99: 1704-1707.
Witomska M Lukazewska AJ (1997). Bulblet regeneration *in vitro* from different explants of *Fritillaria imperialis*. Proc. Int. Symp. on Flower Bulbs. Eds. Lilien-Kipnis H, Halevy AH, Borochov A, Acta Hortic. 430: 331-338.
Witomska M (2000). Effect of temperature on the *in vitro* propagation *Fritillaria imperialis*. Zeszyty naukowe instytutu sadownictwa I kwiaciarstwa w skierniewicach 7: 343-348.
Xie DY Hong Y (2001). Regeneration of *Acacia magnum* through somatic embryogenesis. Plant Cell Rep. 20: 34-40.
Yeung EC (1999). The use of histology in the study of plant tissue culture systems-some practical comments. *In Vitro* Cell Dev. Biol. Plant, 35: 137-143.