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BRIEF COMMUNICATION

Morpho-histological study of direct somatic embryogenesis in endangered species *Frittilaria meleagris*

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Abstract

Direct somatic embryogenesis of *Frittilaria meleagris* L. was induced using leaf base explants excised from *in vitro* grown shoots. Somatic embryos occurred at the basal part of leaf explants 4 weeks after culture on a Murashige and Skoog (MS) medium supplemented with various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) or kinetin (KIN). The highest number of somatic embryos (SEs) were formed (9.74) from leaf explant on MS medium supplemented with 0.1 mg dm^{-3} 2,4-D after 4 weeks of culture initiation. An initial exposure to a low concentration of KIN in the medium also enhanced SEs induction. Our observations by light and scanning electron microscopy revealed that SEs originate directly from the epidermal and subepidermal layers of leaf explant. The developmental stages of somatic embryogenesis from the first unequal cell division through the meristematic clusters, multi-cellular globular somatic embryos to the fully formed cotyledonary embryos were determined. After 4 weeks on MS medium without plant growth regulators, SEs developed into bulblets.

Additional key words: auxin, bulblets, cytokinin.

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Fritillaria meleagris L. is a rare perennial plant, belonging to *Liliaceae* family mainly distributed throughout temperature climates of the Northern Hemisphere. All *Fritillaria* species contain variety of alkaloids with interesting phytochemical properties. They are widely used in traditional Chinese medicine (Li *et al.* 2001). *Fritillaria meleagris* L. is native to Europe, but in many places including France, Slovenia, Romania, as well as Serbia it is an endangered species (Zhang 1983, Ilijanić *et al.* 1998). *F. meleagris* also has great potential as an ornamental plant because of its attractive flowers which open in early spring. Plant production of this species by conventional methods is limited by unpredictable germination of seeds. It is also propagated from bulbs, but only one to two bulbs per year are formed. These facts have resulted in an increased interest for development efficient *in vitro* propagation protocol for this species. Therefore, several *in vitro* culture protocols have been developed in this genus (Sun and Wang 1991, Kukulezanka *et al.* 1998, Gao *et al.* 1999, Paek *et al.* 2002). However, only one among them described somatic embryogenesis in *F. imperialis* (Mohammadani-Dehcheshmeh *et al.* 2007). Somatic embryogenesis has been extensively used as model system to investigate the morphological, biochemical and physiological events of embryogenesis (Rastogi *et al.* 2008, Went dos Santos *et al.* 2008, Vila *et al.* 2009). Reliable plant regeneration through somatic embryogenesis is useful for commercial production, plant breeding, cryopreservation and genetic transformation. Somatic embryogenesis process can be divided into two phases: induction and expression, each of them likely has its own specific hormonal requirement (Jimenéz and Bangerth 2001). The knowledge of developmental stages during somatic embryogenesis achieved by histological study was essential to improve the efficient protocol for *in vitro* propagation in many plants. Somatic embryogenesis was recently obtained in *F. meleagris* in our laboratory (Nikolić *et al.* 2006).

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; FAA - formalin + acetic acid + ethanol; KIN - kinetin; MS medium - Murashige and Skoog medium; SEs - somatic embryos; TDZ - thidiazuron.

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However, there are still doubts concerning the type of origin of these somatic embryos and their further development into whole plants. Thus, the aim of this work was to register the onset of somatic embryogenesis in leaf base culture of *F. meleagris* L. without introducing new plant material in culture.

Stock shoot cultures of *F. meleagris* L. were maintained for several years on Murashige and Skoog (1962; MS) medium supplemented with 3 % (m/v) sucrose, 0.7 % agar and 250 mg dm^{-3} casein hydrolysate, 250 mg dm^{-3} L - proline and 1.0 mg dm^{-3} thidiazuron (TDZ) for shoot multiplication (Nikolić *et al.* 2006). The medium was adjusted to pH 5.8 with 1 M NaOH and autoclaved at 121 °C for 25 min. All cultures were maintained at temperature of 24 ± 2 °C and 16-h photoperiod with irradiance of 40 μmol m^{-2} s^{-1}. Leaf bases segments (about 1 cm in length) taken from the donor plants were transferred to MS medium with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN) for the induction of somatic embryogenesis (Table 1). The percentage of responding explants and the number of somatic embryos (SEs) were counted after 4 weeks in culture. Experiment was performed as five replicates (dishes) containing 10 explants and was replicated three times. The cultures were observed at 5-d intervals for changes in the morphology. The leaf explants were collected 2, 4, 8, 10, 12, 14 and 28 d after culture initiation, divided into small pieces (ca. 5 - 10 mm in length) and fixed using a solution of FAA containing 5.4 cm^3 formalin (37 %), 65.5 cm^3 ethanol (96 %), 5 cm^3 glacial acetic acid and 24 cm^3 distilled water (Jensen 1962). The samples were embedded in *Histowax* (*Histolab*, Göteborg, Sweden). Sections (10 μm) were cut at room temperature using a rotary microtome (*Reichert*, Vienna, Austria). Sections were stretched on a bath of distilled water and mounted on a slide. They were then de-waxed with xylene for 5 - 10 min and then stained with hematoxylin by passing the slides through an ethanol solution [70, 95, 100 % (v/v)] for 15 min each step (Jensen 1962). Sections were mounted in Canada balsam before microscopic examination (*Leica*, *Leitz, DMRB*, Nussloch, Germany). The parts of leaf explants with well developed SEs were observed and photographed under a *JEOL JSM T. 35* (Massachusetts, USA) scanning electron microscope.

First morphological changes in leaf explants were visible after 7 d on all tested media. Early globular SEs have been developed directly on the leaf surface without callus formation (Fig. 1*A*). These SEs can be seen in the vicinity of the leaf cut edge. SEs developing from the distal part of the leaf were less responsive than those developing from the basal part of the same explant, presumably due to differences in physiological state of the cells (Alexandrova and Conger 2002). Mature globular SEs as well as the subsequent stages (Fig. 1*B*) revealed on a leaf surface. The cotyledonary-stages SEs developed within 14 d in culture (Fig. 1*C*). Somatic embryos at different developmental stages were developed on the surface of leaf explants within 28 d of culture (Fig. 1*D*). SEs developed singly and in clusters. The presence of 2,4-D or KIN in the basal medium had a marked effect on direct somatic embryogenesis. Differences were achieved depending on the concentrations tested (Table 1). In the present study, leaf explants showed the highest percentage of SEs induction (93 %) and average number of SEs (9.74) on MS medium supplemented with 0.1 mg dm^{-3} 2,4-D after 4 weeks on culture (Table 1, Fig. 1*D*). In media supplemented with lower concentrations of 2,4-D and KIN (0.1 and 0.5 mg dm^{-3}) we found an improvement production of SEs as compared to other treatments. A higher concentrations of these plant growth regulators delayed somatic embryogenesis. In many species direct somatic embryogenesis from leaf explants have been described on media containing auxins and cytokinins (Wang and Bhalla 2004, Kumar *et al*. 2008). High percentage of SEs induction was also observed for the control culture. Similar results of induction of SEs in medium without plant growth regulators were previously reported in several plants (Subotić and Grubišić 2007, Kumar *et al*. 2008). Many of cotyledonary embryos formed bulblets in the same medium, but number of bulblets produced is low (data not shown). When the explants with cotyledonary SEs were transferred to MS medium lacking plant growth regulators they developed into bulblets. Therefore further study are necessary for synchronize the bulblets development from SEs.

Development of SEs was evaluated by observing leaf explants under scanning electron microscope. About 8 d after induction of somatic embryogenesis, presence of a

Table 1. Effect of plant growth regulators on somatic embryo induction from leaf explants of *F. meleagris* L. Means ± SE of 3 replicates of 50 explants each. Means followed by the same letters within columns are not significantly different according to LSD test at $P \leq 0.05$ probability level.

2,4-D [mg dm^{-3}]	KIN [mg dm^{-3}]	Response [%]	SE number [$explant^{-1}$]
-	-	62	1.23 ± 0.14 a
0.1	-	93	9.74 ± 0.65d
0.5	-	90	8.22 ± 0.51c
1.0	-	79	4.56 ± 0.44b
2.0	-	85	3.91 ± 0.29b
5.0	-	75	2.60 ± 0.24a
10.0	-	65	1.58 ± 0.17a
-	0.1	83	4.91 ± 0.45c
-	0.5	87	5.00 ± 0.41c
-	1.0	82	3.58 ± 0.35b
-	2.0	82	2.68 ± 0.24a,b
-	5.0	63	2.06 ± 0.23a
-	10.0	79	1.99 ± 0.18a

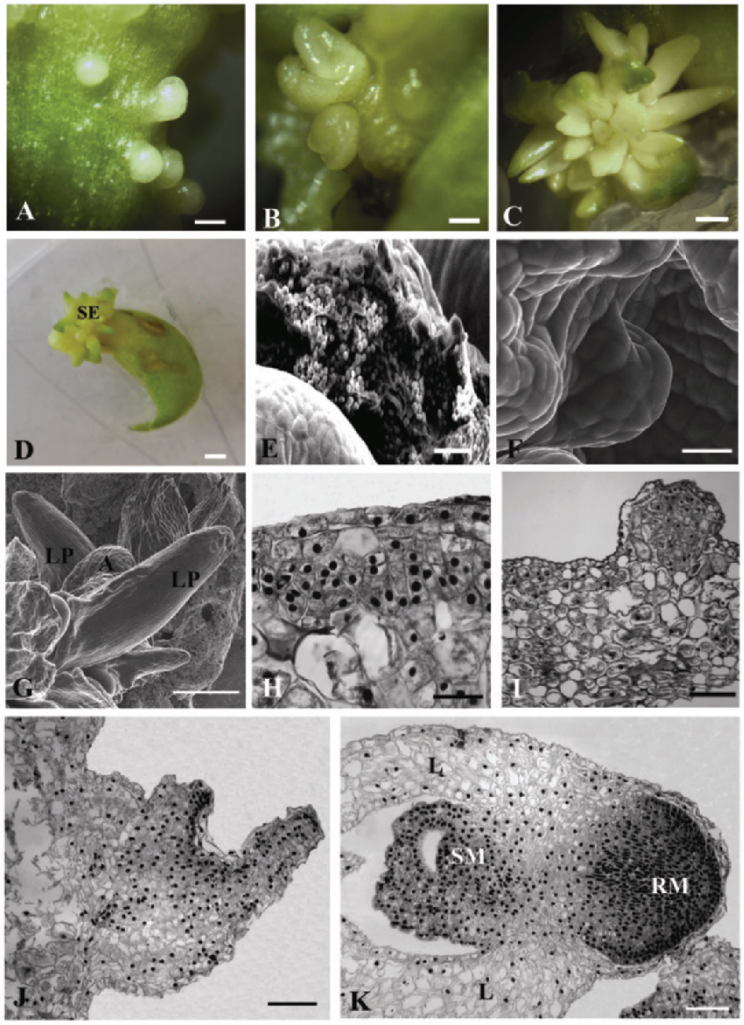

Fig. 1. Somatic embryogenesis induction in leaf base culture of *F. meleagris* cultured on induction medium supplemented with 0.1 mg dm^{-3} 2,4-D. *A* - early globular embryos formed on the surface of leaf explant (*bar* = 0.56 mm); *B* - somatic embryos at the early stage of cotyledonary differentiation (*bar* = 0.3 mm); *C* - clusters of mature cotyledonary somatic embryos developed from the leaf cultured for 14 d (*bar* = 0.9 cm); *D* - leaf explant after 4 weeks of culture with somatic embryos of different developmental stages on the basal cut edge of leaf explant (*bar* = 2 cm); *E* - basal part of leaf surface showing formation of a clusters of dividing cells (*bar* = 500 μm); *F* - globular somatic embryo with protodermal cells after 10 - 12 d of culture (*bar* = 200 μm); *G* - somatic embryos with developed shoot with apex (A) and leaf primordia (LP) (*bar* = 200 μm); *H* -multi-cell embryogenic centers originated due to the periclinal divisions of superficial layers of explant after 8 d in culture (bar = *200* μm); *I* - multicellular origin of proembryogenic centre after 14-d culture (*bar* = 150 μm); *J* - section of early cotyledonary stage embryo with multicellular attachment to the explant after 14 d in culture (*bar* = 50 μm); *K* - longitudinal section of mature cotyledonary somatic embryo showing leaf (L), shoot meristem (SM) and distinctly closed root pole meristem (RM) (*bar* = 200 μm).

prominent cell layer was observed at surface of leaf explants (Fig. 1*E*). Globular SEs were observed 10 - 12 d after initiation of culture (Fig. 1*F*). From SEM observation, SEs maturation were evident on leaf surface about 28 d after initiation of culture (Fig. 1*G*). The SEs maturation appeared to be not well synchronized, as different developmental stages of SEs were observed at the same time. Such direct development of SEs has also been observed in many other plants (Mandal and Datta 2005, Chen and Chang 2006, Liu *et al.* 2007). Auxins and cytokinins are considered to be key factor in the acquisition of embryogenic competence in somatic embryogenesis inductions (Feher *et al*. 2003). The choice of explants for establishment of *in vitro* culture is largely dictated by the method to be adopted for *in vitro* propagation (Mingozzi and Morini 2009). The ability of leaf explants to produce somatic embryos on induction medium clearly indicates that leaves are suitable material for *in vitro* regeneration of *Fritillaria* species. Previous literature usually suggested *in vitro* plant regeneration from bulb scales of *Fritillaria* species. Our results can be compared with commonly used bulbs as explants for *in vitro* regeneration. This is important from several reasons – bulbs are usually contaminated by pathogens;

they have limited number scales and the using of bulbs could be result in the destruction of *Fritillaria* natural populations (Witomska and Lukaszewska 1997). Somatic embryogenesis is the process of *in vitro* regeneration in which bipolar structures are formed from somatic cells without vascular connection with mother tissue. Therefore it is important to identify stages in this process through morphological and histological characteristics. In this study, morphological observations were supported by histological sections of SEs at all particular stages. At the beginning of culture, section of control leaf explants consisted of the leaf's vascular bundles surrounded by parenchyma limited on both sides by an epidermis. Large intracellular spaces occur among palisade cells as a characteristic of *in vitro* grown plants. The first histological changes in leaf explants indicating the development of SEs were visible after 8th day of the culture in epidermal cells, followed by an increase in shape and size of some cells. The enlarging was probably the results of these cell layers being divided in anticlinal direction (Fig. 1*H*). Continuous divisions in all planes give to small proembryogenic clusters. During the whole culture period, dedifferentiation of explant cells continued indicated that the somatic embryogenesis is asynchronous. The cells within the proembryogenic clusters have small size with dense cytoplasm and small nuclei (Fig. 1*I*). During next 8 - 10 d these cells underwent cell division forming SEs at early stage of development. The cells of globular SEs contained small cytoplasm rich cells surrounded by a layer of protoderm cells. Quiroz-Figueroa *et al.* (2006) mentioned that the development of the protoderm is one of unique features of somatic embryo development. The SEs enlarged by sustained divisions. Vascular tissue differentiated later in these structures but it was never connected with initial explant. The development of SE followed by appearance of cotyledons, indicating the early cotyledonery stage (Fig. 1*J*). Mature SEs had well developed cotyledons with leaf primordia at the shoot pole and a well developed root pole (Fig. 1*K*). Histological study revealed that they had bipolar structures and distinct physiological isolation from mother tissues. The epidermis and subepidermal layers of leaf are the places of the origin of cells forming SEs in *F. meleagris*. These meristematic cell aggregates were defined by Haccius (1978) as pro-embryonary cell complexes, and are characteristic of direct embryogenesis. Direct somatic embryogenesis could be suitable model system for the study of histological aspects of the embryogenic transition of somatic cells. To our knowledge, this is the first paper describing induction of direct somatic embryogenesis in *F. meleagris* leaf base culture, on morpho-histological level. In conclusion, this protocol may be useful for conservation of germplasm of endangered *F. meleagris*.

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