IN VITRO BULBLET PRODUCTION AND PLANT REGENERATION FROM IMMATURE EMBRYOS OF *FRITILLARIA TUBIFORMIS* GREN. & GODR.

Valentina Carasso[1] and Marco Mucciarelli[2]*

[1]Natural Park of the Marguareis Massif, 34 S. Anna Road, 12013 Chiusa di Pesio (CN), Italy
[2]Department of Life Sciences and Systems Biology, University of Torino,
25 P. A. Mattioli Blvd, I-10125 Torino, Italy, *Fax +39 011 2369144, *E-mail: marco.mucciarelli@unito.it

Abstract

Fritillaria tubiformis Gren. & Godr. is an alpine geophyte endemic to mountain prairies of the Southwestern Alps. To satisfy the need for *ex situ* propagation of this rare species, a simple and efficient protocol suitable for *in vitro* plant regeneration was developed. Immature zygotic embryos (IZEs) were employed as explant source of somatic embryos (SEs). In consideration of the low temperature requirement for germination of *F. tubiformis*, we studied whether cold stratification (30, 60, and 90 days of incubation at 4°C) affected *in vitro* bulblet regeneration. After one month of culture on MS medium, 30 and 60-day cold stratified IZEs begun to dedifferentiate, in contrast 90-day cold stratified embryos died. Callus proliferation was optimal in the presence of plant growth regulators in the medium. The number of somatic embryos positively correlated with callus, however it was more affected by the length of cold stratification than by the presence of exogenous plant growth regulators in the medium. The maximum number of SEs occurred in 30-day cold stratified zygotic embryos on MS2 medium. Transfer onto a maturation medium supplemented with 4% (w/v) sucrose was essential for the conversion of SEs into bulblets. In the presence of 4.92 μM IBA, bulblets sprouted and rooted and, newly established plants were available for pot cultivation. Greenhouse hardened plants were transferred to open air.

Key words: bulb formation, germination, plant regeneration, somatic embryogenesis

INTRODUCTION

Fritillaria tubiformis Grenier and Godron (1855) is a geophyte endemic to the mountain prairies of the Southwestern Alps (Conti et al. 2005). This plant has beautiful dark-purple flowers, and as many species of the genus, it is cultivated as an ornamental, and sometimes intensively harvested.

Although *F. tubiformis* occurs sporadically in most of the Western and Central Alps, it is very rare and is now protected in the Italian regions where it grows. This plant has been included in the list of regionally threatened European native plant species by Botanic Gardens Conservation International (Sharrock and Jones 2009) and in the virtual seed database of Ensconet (European Native Seed Conservation Network; http://ensconet.maich.gr/main.htm).

Population size restriction due to habitat loss is presently the main concern of extinction to *F. tubiformis* populations, especially in populations that already show limited gene flow due to range contraction and geographical isolation (Mucciarelli and Fay 2013, Mucciarelli et al. 2014).

Germplasm storage of *F. tubiformis* via seed banking is critical for the long-term conservation of this species. In a study run on *F. tubiformis* subsp. *moggridgei* Rix (Carasso et al. 2012) a close relative, endemic to the Maritime and Ligurian Alps, we showed that seeds in these alpine *Fritillaria* are morphologically dormant. In fact, they are dispersed in late summer when embryos are still underdeveloped. Dormancy breaking and germination occur during winter when low temperatures trigger cell mitosis reactivation and zygotic embryo development up to germination in spring (Carasso et al. 2011, 2012). Past attempts to cryopreserve seeds of *F. tubiformis sensu lato* (after Bartolucci and Peruzzi 2012) were unsuccessful. This is probably due to the low level of differentiation reached by immature zygotic embryos (IZEs) at the time of dispersal when seeds are normally dried and stored in the seed bank (Carasso, unpublished). *Ex situ* long-term conservation of *F. tubiformis sensu lato* seeds is, therefore, critical.

In vitro propagation methods are powerful tools for germplasm conservation and propagation of rare and

endangered species (Reed et al. 2011 and references therein).

To date, *in vitro* culture of *F. tubiformis* has not yet been reported. Plant regeneration has been achieved in *F. unibracteata*, *F. thunbergii*, *F. persica*, *F. imperialis*, and in *F. meleagris*. In these species, callus cultures were obtained from bulb fragments (Gao et al. 1999), adventitious bulbils (Kukulczanka et al. 1989), bulbs scales (Paek and Murthy 2002), leaf base explants (Nikolić et al. 2008, Subotić et al. 2010), tepals (Mohammadi-Dehcheshmeh et al. 2008), and mature embryos (Mohammadi-Dehcheshmeh et al. 2006, Petrić et al. 2011). In none of these *Fritillaria* species, however, plant regeneration was obtained from IZEs after cold stratification. Besides, most of the explants employed so far, if taken from nature would be totally or partially destructive for the populations of *F. tubiformis,* considering also the small size of this plant. In search for alternative and more responsive explants, IZEs extracted from seeds are an extremely profitable source of somatic embryos (Parmaksiz and Khawar 2006). The use of zygotic embryos has been exploited in regeneration of food plants (Ćosić et al. 2013, Uma et al. 2012), ornamental species (Clarke et al. 2006, Jevremović et al. 2013), and conifers (Ma et al. 2012).

Considering the importance of establishing suitable propagation techniques of *F. tubiformis*, the culture protocol presented in this study allowed for the *in vitro* multiplication of bulblets starting from seeds collected in the field, cold-conditioned as to reach an optimal level of maturation and cryopreserved in the local seed bank. Subsequently, optimal media for the sprouting and rooting of these bulblets were determined.

MATERIALS AND METHODS

Seed disinfection and cold stratification

Seeds of *F. tubiformis* subsp. *tubiformis* (Jelitto® item No. FA032) were surface-disinfected for 6 min. with 2% (v/v) sodium hypochlorite solution and aseptically rinsed three times with sterile distilled water. Fifteen repetitions of 20 seeds each were sown on 1% agar medium (w/v) in 90 mm glass Petri dishes and incubated at 4°C in the dark for 30, 60, and 90 days of incubation (referred as cold stratification). A further 50 seeds were incubated as above for 90 days, thereafter transferred to $10/20 \pm 2°C$ in a 12/12 h dark/light photoperiod in order to test for germination. This period of stratification was known to be optimal for the embryo to reach the critical length for germination (radicle emergence) (Carasso et al. 2011). At the end of cold stratification, seeds were processed for a further disinfection by immersion in 70% (v/v) ethanol for 3 min, then in 3% (v/v) sodium hypochlorite solution with the addition of a drop of 0.01% Tween 20 (v/v) for 6 min. Thereafter, seeds were rinsed several times with sterile distilled water and employed for embryo rescue.

Explant source, media, and culture conditions

IZEs were aseptically rescued gently squeezing the seed coat with a scalpel and forceps until embryos were released from the micropylar region of the seed (Mirici et al. 2005). Thereafter, one hundred embryos for each of the three cold stratification treatments were transferred to MS medium (Murashige and Skoog 1962), solidified with 10 g l⁻¹ agar, and supplemented with N⁶-benzyladenine (BA) and α-naphthaleneacetic acid (NAA) in different proportions: 0.0 μM BA and 0.0 μM NAA (MS0), 4.44 μM BA and 2.68 μM NAA (MS1), 8.88 μM BA and 2.68 μM NAA (MS2), 13.32 μM BA and 5.36 μM NAA (MS3). These variants of the medium were named "induction" media and their pH was adjusted to 5.7 ± 0.1 with 1 M KOH before autoclaving (121°C for 20 min). The cultures were grown in a cultivation chamber at $24 \pm 2°C$ with 16 h of cool white fluorescent light at photosynthetic photon flux density (PPFD) 90 μmol s⁻¹ m⁻², daily. After 90 days of culture, to promote SEs and bulblet development, zygotic embryos were transferred to a MS medium containing 4 g l⁻¹ sucrose, 4.44 μM BA added to the medium before autoclaving and solidified with 10 g l⁻¹ agar. This medium was named "maturation" medium (MS-M).

To induce rooting and sprouting of the regenerated bulblets, eight months old cultures were transferred to 20 mm × 150 mm borosilicate glass tubes sealed with plastic closure caps and containing 40 ml each of a half-strength MS medium supplemented with full-strength vitamins, 30 g l⁻¹ sucrose, 4.92 μM indole-3-butyric acid (IBA), and solidified with 10 g l⁻¹ agar (pH = 5.7 ± 0.1; "regeneration" medium, MS-P). In order to promote further development of bulblets, stocks of regenerated plants were maintained *in vitro* in 8 cm diameter glass jars kept aerated by a cotton plug inserted in the centre of the metal screw cap and containing 160 ml fresh MS basal medium (MS0).

The bulblets were grown at $10/20 \pm 2°C$ in a 12/12 h dark/light photoperiod of cool white fluorescent light at PPFD 20 μmol s⁻¹ m⁻², daily.

Estimation of growth parameters during plant regeneration

Regeneration efficiencies of embryo cultures were analyzed after 30 and 90 days of culture on the induction medium, after 240 days on the maturation medium and after 60 days of culture on the regeneration medium.

Explanting efficiency was expressed as the number of viable zygotic embryos over the number of total explants and as a percentage calculated dividing the number of viable explants to the total number of explants × 100. Callus production was expressed as the number of callus over the number of total explants and callus induction frequency as a percentage calculated dividing the number of calluses to the total number of viable explants × 100. Embryogenic efficiency was

expressed as the number of somatic embryos over the number of total explants after 240 days of culture on the maturation medium (MSM). Embryo induction frequency was calculated as ratio of the number of somatic embryos to the total number of viable explants × 100. In fact, somatic embryos developed either from callus or directly on dedifferentiating zygotic embryos. Regeneration efficiency was expressed as the number of regenerated bulblets over the number of total explants after 60 days of culture on the regeneration medium (MS-P). Bulblet induction frequency was obtained dividing the number of bulblets to the total number of somatic embryos × 100.

In seeds, which have moved to warmer temperatures after 90 days of cold stratification, mean germination percentage was recorded after 120 days of incubation. Seedling development up to bulb initiation was documented and the main steps of this process photographed using a Canon EOS 1100D digital camera (Canon Inc., Tokyo, Japan).

Statistical analysis

All the experiments were carried out in a completely randomized design. Twenty explants in five replicates for a total of one hundred explants were used for each treatment. Statistical analysis concerned the number of viable zygotic embryos after 30 days on MS induction media, the number of calluses in 90 days cultures, the number of somatic embryos and regenerated bulblets after 240 and 300 days of culture respectively. Percentages of viable explants, calluses, somatic embryos, and bulblets were arcsine transformed before statistical analysis. These data were subjected to two-factor Analysis of Variance (ANOVA) using least squares means and Tukey HSD multiple pairwise mean comparisons calculated at the probability level of $p \leq 0.001$. Pearson correlation coefficients (R) were calculated for the number of calluses, somatic embryos and regenerated bulblets at the probability level of $p \leq 0.001$. The results were analyzed statistically using Systat version 11 for Windows.

RESULTS

Initiation and maintenance of somatic embryogenesis

Incubation on MS induction medium resulted in a considerable increase in length of *F. tubiformis* zygotic embryos. By the time of extraction 30-days and 60-days cold stratified embryos were 1.86 ± 0.12 mm and 2.93 ± 0.12 mm long respectively (Fig. 1A,B) and measured almost 4.0 ± 1.3 mm after 30 days on the induction media at $24 \pm 2°C$ (Fig. 1C). Not all rescued embryos, however, survived. After 30 days of culture, the number of viable explants ranged between 12.78 ± 2.92 and 19.60 ± 2.22 every twenty 30-days and 60-days cold stratified embryos, respectively (Table 1). Percentages

of viable explants varied between $63.9 \pm 14.58\%$ and $98 \pm 11.10\%$, while most of 90-days cold stratified embryos died during the first 30 days of incubation (Table 1). Because of incubation on MS media, the basal portion (towards the root tip) of viable IZEs swelled and protruded laterally (Fig. 1C, simple arrows), meanwhile the root tip elongated (double arrow) and first signs of tissue dedifferentiation took place.

By the end of incubation on the four variants of the induction medium, white and greenish patches of proliferating callus developed on the surface of IZEs (Fig. 1D). Callus induction efficiency varied between $0.10 \pm 0.22\%$ to $73.90 \pm 16.86\%$ according to the medium variant and the explant (Table 2). The analysis of variance showed that the number of viable IZEs, which developed callus was affected by length of cold stratification (30-90 days; ANOVA, F-ratio = 241.42, $p < 0.001$), culture medium composition (MS0/MS3; ANOVA, F-ratio = 108.82, $p < 0.001$) and their reciprocal combination (ANOVA, F-ratio = 48.28, $p < 0.001$). At increasing BA levels up to 8.88 µM the average number of callus increased significantly together with callus induction frequency (induction medium MS2, 30-days and 60-days cold stratified embryos; Table 2). We observed that on average, 9.45 ± 2.15 every twenty 30-days cold stratified embryos developed callus on MS2 ($p < 0.001$). In 60-day cold stratified embryos, maximum number of calluses ranged between 5.83 ± 0.69 and 8.33 ± 0.99 on MS1 and MS2, respectively (Table 2). Based on Least Squares means of average callus numbers, no significant differences between the first two cold treatments were registered (Fig. 2A). On the contrary, very few callus was obtained from 90-days cold stratified embryos (0.10 ± 0.22 and $0.50 \pm 0.50\%$, $p = 0.470$) (Table 2, Fig. 2A). The analysis of variance (Tukey HSD Multiple comparison) showed that callus formation was significantly enhanced in the presence of 8.88 µM BA and 2.68 µM NAA (MS2) (Fig. 2B).

After one month of culture on the maturation medium (MS-M), somatic embryos (SEs) developed on 30-days and 60-days cold stratified IZEs (Fig. 1D,E); the number of SEs continued to increase during the following months. According to the analysis of variance, numbers of somatic embryos were more affected by length of cold stratification (30-90 days; ANOVA, F-ratio = 256.61, $p < 0.001$), than by culture medium composition (MS0/MS3; ANOVA, F-ratio = 97.08, $p < 0.001$) or the reciprocal combination of the two factors (ANOVA, F-ratio = 67.78, $p < 0.001$). Indeed, somatic embryo induction frequency was higher in 30-days cold stratified embryos, and ranged from $54.2 \pm 4.06\%$ to $578.26 \pm 131.92\%$ ($p < 0.001$, Table 3). The latter figure corresponded to an average of 73.90 ± 15.08 somatic embryos developed from 12.78 ± 2.92 viable zygotic embryos (Table 1-3). Concerning culture medium variant, we observed that, on average, 2.8 and 5.8 somatic
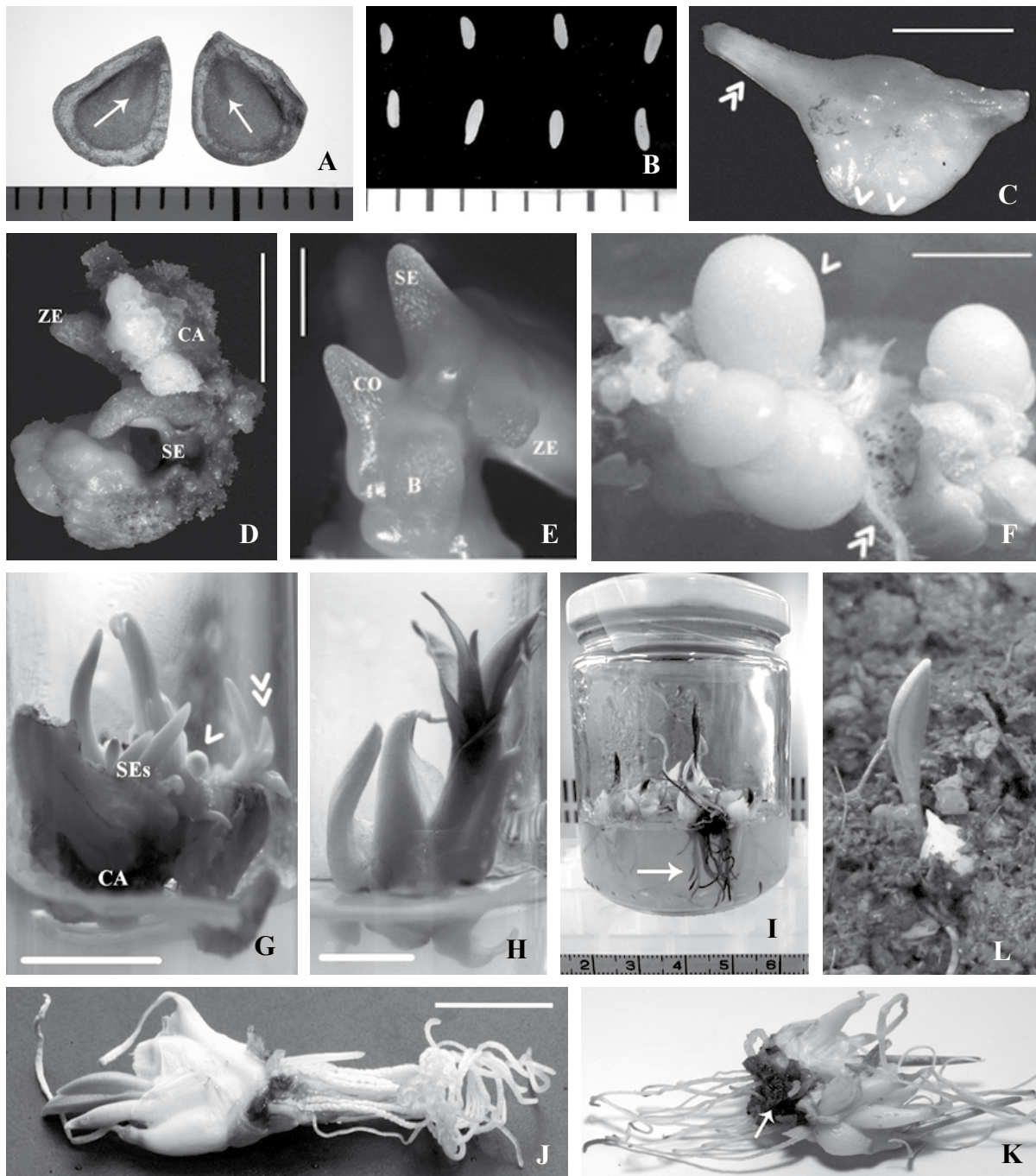
Fig. 1. Tissue culture of *F. tubiformis*: bulblet development and plant regeneration. A) Fresh seeds after 30 days of cold stratification; the growing embryo is visible inside (arrows). Bar = 10 mm, B) Immature zygotic embryos (IZEs) excised from fresh seeds after 30 days of incubation at 4°C. Bar = 10 mm, C) A 30 days cold stratified IZE of *F. tubiformis* after 1 month of culture on the induction medium (MS0). Bar = 2 mm, D) Callus proliferation on MS2 medium. ZE: zygotic embryo; SE: somatic embryo; CA: callus. Bar = 5 mm, E) Somatic embryos (SEs) emerging from the surface of an IZE (ZE), after four months of culture (MS-M medium); CO = cotyledon, B = bulblet primordium. Bar = 1 mm, F) Bulblets with roots (double arrows) developed over a 8-month-old explant on MS-M medium. Bar = 3 mm, G) Multiplication of somatic embryos (SEs) and new bulblets (single arrow) on the regeneration medium (MS-P) supplemented with 4.92 μM IBA. A newly emerging shoot is also visible (double arrow). Callus (CA) at the base of the explant. Bar = 10 mm, H) One of the shoots showing white, thick scales at the base. Bar = 10 mm, I) Sprouting bulblets with roots (arrow) and green shoots on maintenance medium. Bar = 10 mm, J-K) Clamps of regenerated plantlets of *F. tubiformis*. Arrow indicates the remnant callus. Bar = 10 mm, L) A young plant of *F. tubiformis* after one year on soil at the alpine nursery.

Table 1. Mean numbers of *F. tubiformis* viable zygotic embryos and their percentages after 30 days of culture, according to the length in days of cold stratification at 4°C and the variant of induction medium.

BA (μM)	NAA (μM)	Number of viable explants			Viable zygotic embryos (%)		
		30 days	60 days	90 days	30 days	60 days	90 days
0	0	19.60 ± 1.14 a A	15.84 ± 1.01 ab B	0.11 ± 0.15 a C	98.00 ± 5.70 a A	79.20 ± 5.05 a B	0.53 ± 0.74 a C
4.44	2.68	13.33 ± 1.58 b A	13.33 ± 1.58 a A	0.10 ± 0.14 a B	66.70 ± 7.91 b A	66.70 ± 7.91 b A	0.50 ± 0.71 a B
8.88	2.68	12.78 ± 2.92 b A	19.60 ± 2.22 b B	0.11 ± 0.15 a C	63.90 ± 14.58 b A	98.00 ± 11.10 a B	0.53 ± 0.74 a C
13.32	5.36	13.33 ± 1.00 b A	16.90 ± 2.73 ab A	0.16 ± 0.36 a B	66.65 ± 5.00 b A	84.50 ± 13.66 b A	0.80 ± 1.79 a B
p		<0.001	0.003	0.968	<0.001	0.001	0.968

Means ± standard deviation followed by the same lower case letter in a column within a group are not significantly different according to Duncan's multiple range test at $p \leq 0.05$. Means followed by the same capital letter in a row within a group are not significantly different according to Duncan's multiple range test at $p \leq 0.05$.

embryos developed from the surface of a single 30-days cold stratified embryo after eight months on MS0 and MS2, respectively. Based on ANOVA results (Table 3) and their Least Squares means (Fig. 2D), however, no significant differences were registered between the mean number of somatic embryos obtained with these two medium variants. In 60-days cold stratified embryos, somatic embryo induction frequency was on average significantly lower (Fig. 2C), with a minimum of 19.05 ± 3.08% (MS3) and a maximum of 268.75 ± 31.88% SEs (MS1) (Table 3). Generally, on MS3 medium, the mean number of SEs was always low, ranging from 0.05 ± 0.06 and 7.22 ± 0.48 (Table 3) and very few of them developed on 90-days cold stratified embryos (Table 3, Fig. 2C). According to Pearson's coefficients the number of somatic embryos positively correlated with the number of calluses that developed on 30-days and 60-days cold stratified IZEs (R = 0.993-0.899 respectively, $p \leq 0.001$; Fig. 3) cultured on induction media.

Proliferation of bulblets

On maturation medium, SEs gradually converted into bulblets. Bulblets arose as growing globular masses at the base of the cotyledonary pole of a single SE (Fig. 1E). Older and larger bulblets consisted in a continuous white translucent scale (Fig. 1F); sometimes they de-

veloped one or more rootlets on the same medium (Fig. 1F). When this material was transferred onto the regeneration medium (MS-P), new SEs bulblets developed simultaneously (Fig. 1G). Sporadic shoot formation from callus was also observed on MS-P medium (Fig. 1G); these shoots were excised from the explant and transferred to new MS-P media where they developed thick scales enclosing the shoot apex (Fig. 1H). On MS-P medium, 60-days cold stratified embryos showed the highest bulblets induction frequency (Fig. 2E) with a maximum of 150.00 ± 24.24% of bulblets after SEs induction on MS3 medium (Table 4). This percentage corresponded to an average of 5.00 ± 0.93 bulblets developed from 3.33 ± 0.62 somatic embryos (Table 2-3). In 30-days cold stratified embryos, maximum bulblet induction was also obtained from SEs induced on MS3 medium (76.92 ± 5.77%, Table 4). MS1 and MS2 medium variants, however, returned the highest number of bulblets; for example, an average of 23.30 ± 2.47 and 12.50 ± 1.33 bulblets were obtained from twenty 60-days cold stratified embryos, respectively (Table 4, Fig. 2F). However, according to the results of ANOVA, bulblet numbers were largely affected by length of cold stratification (30-90 days; ANOVA, F-ratio = 418.5, $p < 0.001$), followed by a much lower effect deriving from the combination of this factor and

Table 2. Mean numbers of *F. tubiformis* calluses and callus induction frequency (%) after 90 days of culture, according to the length in days of cold stratification at 4°C and the variant of induction medium.

BA (μM)	NAA (μM)	Number of calluses			Callus induction frequency (%)		
		30 days	60 days	90 days	30 days	60 days	90 days
0	0	5.00 ± 0.40 a A	0.83 ± 0.05 a B	0.02 ± 0.04 a C	25.00 ± 1.45 a A	5.26 ± 0.34 a B	0.10 ± 0.22 a C
4.44	2.68	3.89 ± 0.46 a A	5.83 ± 0.69 b B	0.10 ± 0.10 a C	29.20 ± 3.46 a A	43.80 ± 5.19 b B	0.50 ± 0.50 a C
8.88	2.68	9.45 ± 2.15 b A	8.33 ± 0.99 c A	0.08 ± 0.11 a B	73.90 ± 16.86 b A	41.67 ± 4.72 b B	0.38 ± 0.54 a C
13.32	5.36	0.56 ± 0.04 c A	3.33 ± 0.69 d B	0.06 ± 0.09 a A	4.17 ± 0.31 c A	19.05 ± 3.08 c B	0.30 ± 0.45 a A
p		<0.001	<0.001	0.557	<0.001	<0.001	0.470

Means ± standard deviation followed by the same lower case letter in a column within a group are not significantly different according to Duncan's multiple range test at $p \leq 0.05$. Means followed by the same capital letter in a row within a group are not significantly different according to Duncan's multiple range test at $p \leq 0.05$.
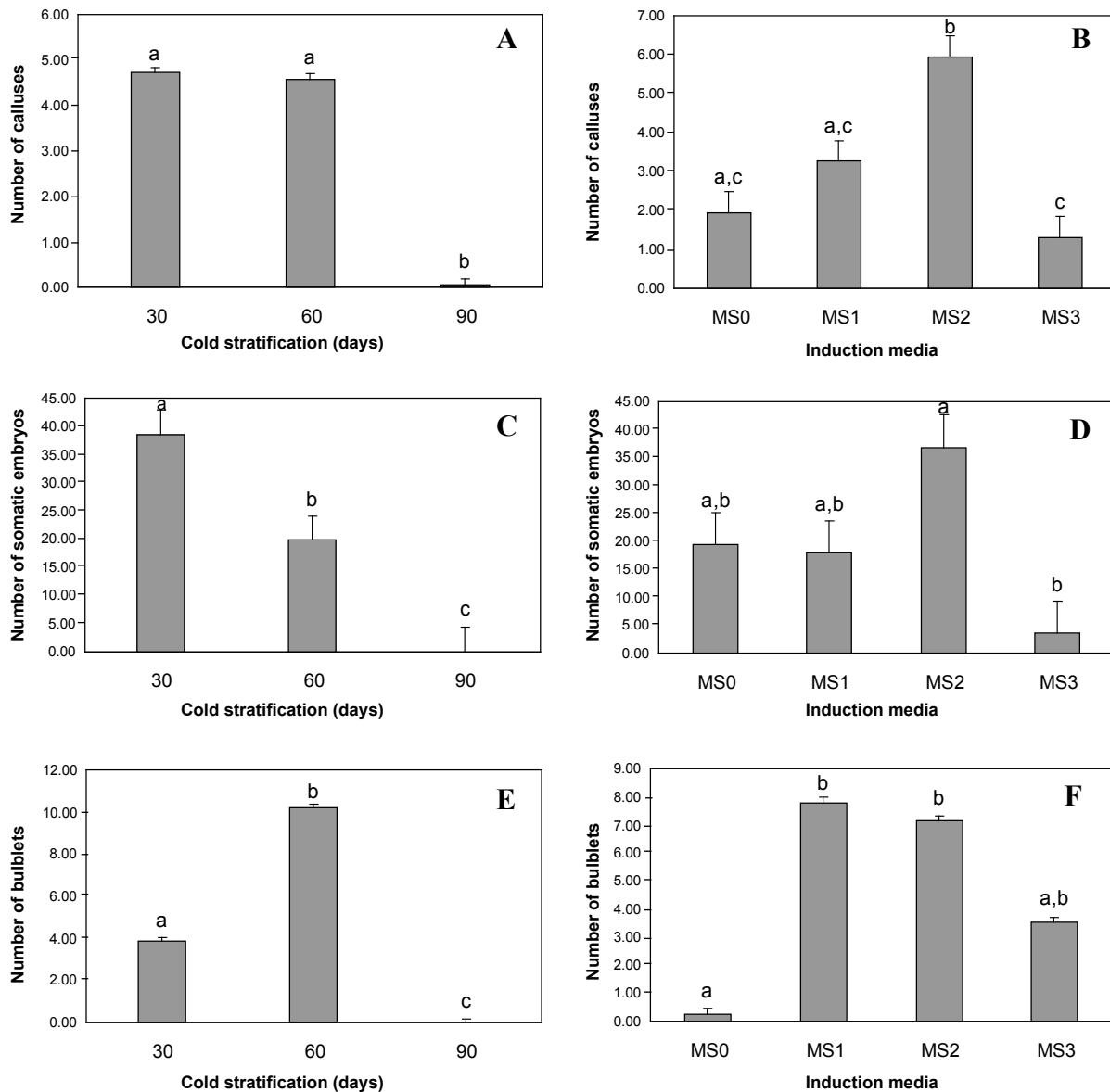
Fig. 2. Effect of cold stratification (A, C, E) and induction media (B, D, F) on the number of calluses, somatic embryos, and bulblets (see plot ordinates) obtained from *F. tubiformis* IZEs. Data represents Least Squares means as obtained from the Two-way ANOVA analysis where the mean number of calluses, somatic embryos, and bulblets (dependent variables) are plotted for each level of the two factors (cold stratification and culture medium variants). Means followed by the same letter are not significantly different at $p < 0.05$ according to the Post Hoc Test.

culture medium variant (ANOVA, F-ratio = 166.1, $p < 0.001$) and from medium composition alone (ANOVA, F-ratio = 140.0, $p < 0.001$). In fact, differences in the number of regenerated bulblets among variants of induction medium originally applied to IZEs received little statistical support (Fig. 2F). No bulblets regenerated from 90-days cold stratified embryos (Table 4, Fig. 2E). In general, *in vitro* bulblets of *F. tubiformis* were positively correlated with either calluses or somatic embryos; however, Pearson's correlation coefficients were high and statistically supported ($R = 0.716$ and $R = 0.871$, bulblets vs. calluses and somatic embryos

respectively; $p \leq 0.001$) only in bulblets obtained from 60-days cold stratified IZEs.

Regeneration of plantlets

Long permanence (more than two months) on MS-P medium was detrimental and bulblets started to whiter and finally decayed (data not shown). On the contrary, once transferred to larger vessels containing MS basal medium (MS0) and grown at climatic chamber conditions, bulblets rooted (Fig. 1I, arrow) and sprouted to give two or three leaves (Fig. 1I, J, K). Supplemental leaf scales developed at the base of the bulblet enclos-

Table 3. Mean numbers of *F. tubiformis* somatic embryos and embryo induction frequency (%) after 240 days of culture on the maturation medium (MS-M), according to the length in days of cold stratification at 4°C and the variant of induction medium.

BA (µM)	NAA (µM)	Number of somatic embryos			Embryo induction frequency (%)		
		30 days	60 days	90 days	30 days	60 days	90 days
0	0	55.00 ± 3.89 a A	3.33 ± 0.19 a B	0.06 ± 0.08 a B	275.00 ± 16.00 a A	21.05 ± 1.34 a B	0.30 ± 0.45 a C
4.44	2.68	17.77 ± 1.89 b A	35.82 ± 3.80 b B	0.08 ± 0.07 a C	133.33 ± 15.82 a A	268.75 ± 31.88 b B	0.40 ± 0.42 a C
8.88	2.68	73.90 ± 15.08 a A	36.6 7 ± 3.89 b B	0.00 ± 0.00 a C	578.26 ± 131.92 b A	183.33 ± 20.76 c B	0.00 ± 0.00 a C
13.32	5.36	7.22 ± 0.48 c A	3.33 ± 0.62 a B	0.05 ± 0.06 a B	54.17 ± 4.06 c A	19.05 ± 3.08 a B	0.24 ± 0.35 a C
p		<0.001	<0.001	0.352	<0.001	<0.001	0.352

Means ± standard deviation followed by the same lower case letter in a column within a group are not significantly different according to Duncan's multiple range test at $p \leq 0.05$. Means followed by the same capital letter in a row within a group are not significantly different according to Duncan's multiple range test at $p \leq 0.05$.

ing the green elongating leaves (Fig. 1J, K). Sometimes hard brown callus masses were still visible at the base of these leaf scales (Fig. 1K, arrow).

After 1 year the plantlets were successfully transferred to soil in experimental plots at the E. Burnat & C. Bicknell Alpine Botanic Station (CN) on 1970-2000 m a.s.l. (Fig. 1L, data not shown).

Seed germination and bulb development

On average, 70% of the seeds of *Fritillaria tubiformis* germinated when moved to $10/20 \pm 2°C$ after 90 days of cold stratification. After germination (radicle protrusion), the testa and endosperm of the seed remained attached to the cotyledon tip, while the primary root progressively distended (Fig. 4A,B,C). The cotyledon and the hypocotyl continued to elongate and together reached approximately 3 cm of length after six months of cultures (Fig. 4D). At this stage, a bulb primordium became evident at the base of most of the seedlings (Fig. 4E). In fact, bulbs in *F. tubiformis* first originated as a swelling of tissues positioned at the hypocotyl node of the seedling and very close to the limit of the root hairs (Fig. 4E,F). After almost seven months from germination, the bulb was ovoid, white translucent in surface and reached its final size (3-5 mm on average) (Fig. 4G). In fact, by this time, the primary
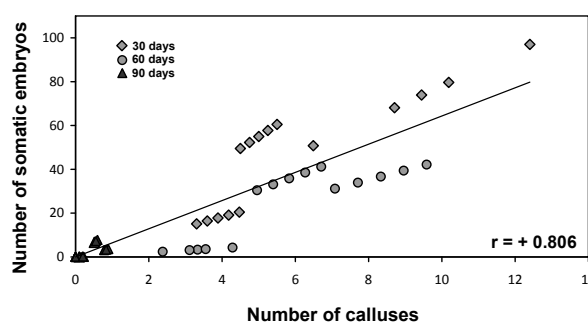
Fig. 3. Relationship (Pearson's correlation, $p \leq 0.001$) of callus production (mean number of calluses per repeat) and somatic embryos (mean number of SEs per repeat, n = 20, on MS-M medium) in *F. tubiformis* IZEs after three different cold stratifications (different symbols indicate differences in days).

roots and the cotyledons of seedlings had degenerated (Fig. 4G), and meanwhile the newly formed bulbs had stopped to grow. After rescue from culture plates, bulbs were successfully stored at cold.

DISCUSSION

In the past, seed banking procedures applied to *Fritillaria tubiformis* Gren. & Godr. failed mostly because of the rapid loss of seed viability during dry storage. It

Table 4. Mean numbers of *F. tubiformis* bulblets and their induction frequency (%) after 60 days of culture on the regeneration medium (MS-P), according to the length in days of cold stratification at 4°C and the variant of induction medium.

BA (µM)	NAA (µM)	Number of bulblets			Bulblets induction frequency (%)		
		30 days	60 days	90 days	30 days	60 days	90 days
0	0	1.11 ± 0.08 a A	0.00 ± 0.00 a B	0.00 ± 0.00 a B	2.02 ± 0.12 a A	0.00 ± 0.00 a B	0.00 ± 0.00 a B
4.44	2.68	0.00 ± 0.00 b A	23.30 ± 2.47 b B	0.00 ± 0.00 a A	0.00 ± 0.00 b A	65.12 ± 7.72 b B	0.00 ± 0.00 a A
8.88	2.68	8.89 ± 1.81 c A	12.50 ± 1.33 c A	0.00 ± 0.00 a B	12.03 ± 2.74 c A	34.09 ± 3.86 c B	0.00 ± 0.00 a C
13.32	5.36	5.55 ± 0.37 d A	5.00 ± 0.93 d A	0.00 ± 0.00 a B	76.92 ± 5.77 d A	150.00 ± 24.24 d B	0.00 ± 0.00 a C
p		<0.001	<0.001	n.d.	<0.001	<0.001	n.d.

Means ± standard deviation followed by the same lower case letter in a column within a group are not significantly different according to Duncan's multiple range test at $p \leq 0.05$. Means followed by the same capital letter in a row within a group are not significantly different according to Duncan's multiple range test at $p \leq 0.05$.
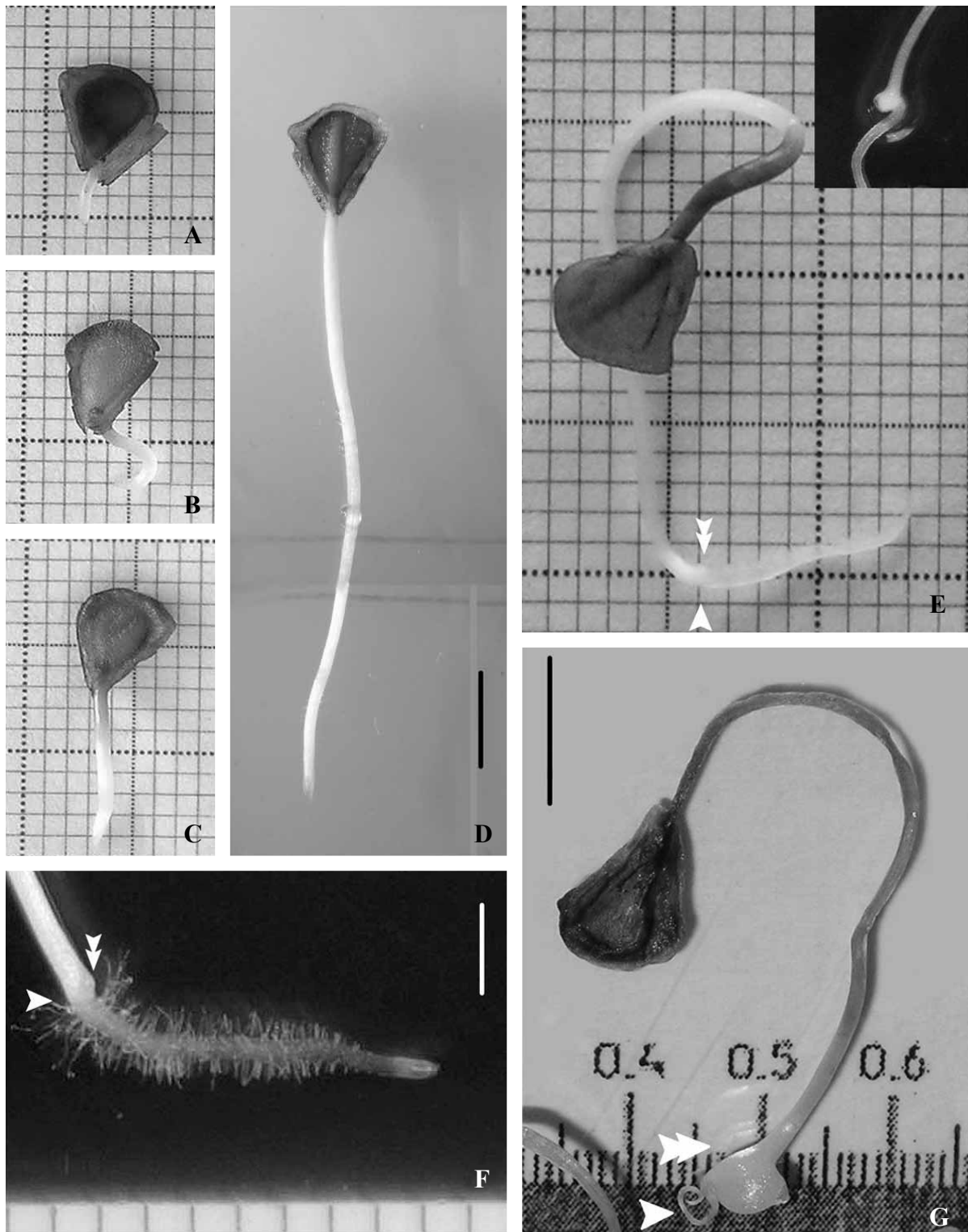
Fig. 4. Germination of *Fritillaria tubiformis* at 10/20 ± 2°C in a 12/12 h dark/light photoperiod, after 3 months of cold stratification. A) Primary root protrudes from seeds, the cotyledon and the hypocotyl elongate and start to green (14 days, B and one month later, C). (D) A mature seedling of *F. tubiformis* after two months of culture, showing a full elongated cotyledon (metric bar = 5 mm), E) Another seedling of the same age already showing a bulb primordium (double arrows) at the hypocotyl node (simple arrow). A magnification of the primordium (inset at the upper right), F) A detail of the primary root and of the hypocotyl zone with the developing bulb primordium (double arrows) (metric bar = 2 mm); root hairs (single arrow), G) A seedling of *F. tubiformis* after 7 months of culture; the bulb has enlarged to almost its final size (double arrow). Note also degenerating root (single arrow) and the withering of cotyledon (metric bar = 11.5 mm).

was evident, therefore, that a single conservation strategy for this endemic species was unlikely to achieve the expected conservation goals.

For this reason, we reported a protocol for tissue culture propagation of *F. tubiformis* starting from cold conditioned immature zygotic embryos (IZEs) and showed that *in vitro* plant regeneration of this alpine endemic is feasible.

Thanks to this culture protocol, embryos can be rescued from old seed accessions of *F. tubiformis*, cultured on nutrient media and employed for plant regeneration. In this paper, we showed also that three months at 4°C are necessary for germination of *F. tubiformis* seeds. Considering that low temperatures were shown to prompt cell growth reactivation in resting embryos of *F. tubiformis* subsp. *moggridgei* almost immediately after cold exposure (Carasso et al. 2012), we used cold-conditioned seeds of *F. tubiformis* as initial explants and tested whether different cold pretreatments were able to affect their response to *in vitro* explanting and culture.

We demonstrated that *F. tubiformis* explants of IZEs cold-conditioned for 30 or 60 days represent a good source of somatic embryos and regenerated bulblets. The procedure is practicable for two main reasons. Firstly, IZEs can be axenically isolated from seeds, while other responsive explants (i.e. bulb scales in Kedra and Bach 2005) are frequently contaminated by bacteria and fungi (Mohammadi-Dehcheshmeh et al. 2008). Secondly, because IZEs of *F. tubiformis* maintain a high intrinsic embryogenic potential for relatively long periods. This has been also documented in other species (Elhiti and Stasolla 2011, Uma et al. 2012) and it is consistent with the observation that IZEs normally contain a high number of undifferentiated proembryogenic cells (Neelakandan and Wang 2012 and references therein) which number declines during the course of embryo maturation (Garrocho-Villegas et al. 2012). Our results showed, in fact, that the first event of tissue dedifferentiation in *F. tubiformis*, a prominent lateral swelling of IZEs protoderm (Fig. 1C), was intrinsically dependent on the developmental stage reached by IZEs at the time of explanting and, almost unaffected by the application of plant growth regulators (Table 1).

Somatic embryos (SEs) development in *F. tubiformis* declined proportionally with increasing durations of cold stratification (Fig. 2C) being maximum after 30-days cold stratification and, the number of these structures positively correlated with IZEs dedifferentiation ($R = 0.806$, $p < 0.001$, Fig. 3). A similar finding has been documented, for example, in tissue cultures of *Carya cathayensis* Sarg. where frequency of SEs increased with age of the zygotic embryos up to 11 weeks after pollination and decreased consistently thereafter (Zhang et al. 2012). These results might also depend by the amount of callus proliferating on the explants. In *F. tubiformis*, MS2 medium supplemented with 2 mg

l^{-1} BA and 0.5 mgl^{-1} NAA proved to be superior both in terms of number of calluses obtained and of SEs (Fig. 2B, D), however, callus proliferation approximately occurred only in 50% of IZEs producing SEs (Table 2). In this regard, SEs were abundant also on MS0 medium given that 30 days cold stratified IZEs were employed as source explants. MS3 medium having a BA/NAA ratio of 0.4 returned the lowest number of SEs (Fig. 2D).

Among the factors involved in the regulation of the embryogenic potential of tissues having different age and level of differentiation, are endogenous growth regulators (Rode et al. 2012). Levels of abscisic acid (ABA), for example, declines during initial stages of embryo development, to increase again along with seed dormancy onset at mature embryo stages (Santa-Catarina et al. 2006). This finding seems to imply a role of this PGR and other growth regulators in tissue recalcitrance to cell dedifferentiation and in the low SE efficiency observed in 90-days cold conditioned IZEs. In *F. tubiformis*, the involvement of ABA in somatic embryogenesis and its dependence from cold stratification of IZEs seems obvious.

Our results showed that in *F. tubiformis* also the process leading to bulblet regeneration was affected by the level of maturation reached by the IZEs at the time of explanting. As expected from the low number of explants dedifferentiating on induction medium, 90 days cold stratified IZEs produced a few calluses, the number of SEs was very low and no bulblets were generated (Fig. 2A,C,E). ANOVA results supported these findings and showed that plant regeneration was affected by the developmental level reached by IZEs at the time of explanting rather than from the culture medium variant. According to what we have previously documented in *F. tubiformis* subsp. *moggridgei*, size and morphology of zygotic embryos may change dramatically during cold stratification of seeds (Carasso et al. 2011) and presumably also their embryogenic competence. Similar events may have occurred during cold stratification of seeds employed for this study and before explanting; we argue that the morphogenetic and biochemical processes involved in seed dormancy breakage during cold pretreatment might have dramatically marked the progressive loss of *in vitro* regenerative potential.

In support of this finding, we have also documented that *F. tubiformis* bulblets developed directly from somatic embryos. This was also documented by Mirici et al. (2005) in *S. fischeriana* where, however, bulblets arose from coleoptile-like structures present on the embryogenic callus. In somatic embryos of *F. tubiformis*, we found similar structures and interpreted them as cotyledon-like (Fig. 1E). This finding is supported also by what we have observed in normal seedlings, where bulbs developed at the base of the cotyledon, and no plumular leaves or cotyledon sheaths were vis-

ible. Seedling development ended when the cotyledon interrupted his trophic function and became shriveled up (Fig. 4E-G). In *Fritillaria* spp., we observed that *in vitro* formation of a bulblet is strictly dependent on the maturation level reached by the zygotic embryo at time of explanting. Under a developmental point of view, we can assume that bulblet morphogenesis from SEs outlines bulb formation in normal seedlings; differing the two, somatic embryo and seedling, for the level of maturation required at the time of differentiation. Both these processes are under the control of low temperatures (Carasso et al. 2012).

According to us, bulblets regeneration in *F. tubiformis* can be regarded as the result of somatic embryogenesis occurring in IZEs. This is supported by the observation that no shoots developed *in vitro* before the bulblet; in this regard, bulblet regeneration corresponds to bulb formation in juvenile seedlings. It has been reported that shoots resembling sprouting bulblets can develop directly from IZEs when no 2,4-D was supplemented to culture media (Mirici et al. 2005, Petrić et al. 2011) and that this PGR is essential for somatic embryogenesis. However, we rarely observed shoots developing directly on dedifferentiated IZEs, and on this occurrence, buds rescued on fresh media never converted into bulblets. Besides, somatic embryogenesis and bulblets in the absence of this synthetic auxin have been documented in tissue cultures of *Fritillaria* spp. (Mohammadi-Dehcheshmeh et al. 2006, 2008, Subotić et al. 2010) and in other species (Parmaksiz and Khawar 2006, Zou et al. 2014), demonstrating that this PGR is not required for SEs induction in geophytes.

REFERENCES

Bartolucci F., Peruzzi L. (2012). Typification of *Fritillaria tubiformis* Gren. & Godr., *Fritillaria delphinensis* f. *moggridgei* Planch. and *Fritillaria delphinensis* var. *burnatii* Planch. (Liliaceae) from SW Europe. Candollea, 67: 23-29.

Carasso V., Hay F. R., Probert R. J., Mucciarelli M. (2011). Temperature control of seed germination in *Fritillaria tubiformis* subsp. *moggridgei* (Liliaceae) a rare endemic of the South-West Alps. Seed Science Research, 21: 33-38.

Carasso V., Fusconi A., Hay F. R., Dho S., Gallino B., Mucciarelli M. (2012). An alpine threatened species, *Fritillaria tubiformis* subsp. *moggridgei*: seed morphology and temperature regulation of the embryo growth. Plant Biosystems, 146: 74-83.

Clarke H. J., Wilson J. G., Kuo I., Lulsdorf M. M., Mallikarjuna N., Kuo J., Siddique K. H. M. (2006). Proteomic and histological analyses of endosperm development in *Cyclamen persicum* as a basis for optimization of somatic embryogenesis. Plant Cell, Tissue and Organ Culture, 85: 197-204.

Conti F., Abbate G., Alessandrin A., Blasi C. (2005). An annotated checklist of the Italian vascular flora. Palombi, Roma, 420 pp.

Ćosić T., Vinterhalter B., Vinterhalter D., Mitić N., Cingel A., Savić J., Bohanec B., Ninković S. (2013). *In vitro* plant regeneration from immature zygotic embryos and repetitive somatic embryogenesis in kohlrabi (*Brassica oleracea* var. *gongylodes*). In Vitro Cellular & Developmental Biology-Plant, 49: 294-303.

Elhiti M., Stasolla C. (2011). The use of zygotic embryos as explants for *in vitro* propagation: an overview. Methods in Molecular Biology, 710: 229-55.

Gao S. L., Zhu D. N., Cai Z. H., Jiang Y., Xu D. R. (1999). Organ culture of a precious Chinese medicinal plant – *Fritillaria unibracteata.* Plant Cell, Tissue and Organ Culture, 59: 197-201.

Garrocho-Villegas V., Jesus-Olivera M. T., Quintanar E. S. (2012). Maize somatic embryogenesis: recent features to improve plant regeneration. Methods in Molecular Biology, 877: 173-182.

Grenier M., Godron M. (1855). Notices botaniques sur quelques plantes des genres *Fritillaria*, *Asphodelus* et *Melica*. Mémoires de la Société d'Emulation du Département du Doubs. Besançon, serie 2, 6: 12-15.

Jevremović S., Jeknić Z., Subotić A. (2013). Micropropagation of *Iris* sp. Methods in Molecular Biology, 11013: 291-303.

Kedra M., Bach A. (2005). Morphogenesis of *Lilium martagon* L. explants in callus culture. Acta Biologica Cracoviensia Series Botanica, 47: 65-73.

Kukulczanka K., Kromer K., Czastka B. (1989). Propagation of *Fritillaria meleagris* L. through tissue culture. Acta Horticulturae, 251: 147-154.

Ma X., Bucalo K., Determann R. O., Cruse-Sanders J. M., Pullman G. S. (2012). Somatic embryogenesis, plant regeneration, and cryopreservation for *Torreya taxifolia*, a highly endangered coniferous species. In Vitro Cellular & Developmental Biology-Plant, 48: 324-334.

Mirici S., Parmaksiz I., Özcan S. Sancak C., Uranbey S., Sarihan O., Gümüşcü A., Gürbüz B., Arslan N. (2005). Efficient *in vitro* bulblet regeneration from immature embryos of endangered *Stenbergia fischeriana*. Plant Cell, Tissue and Organ Culture, 80: 239-246.

Mohammadi-Dehcheshmeh M., Khalighi A., Ebrahimie E., Sardari M., Naderi R. (2006). Direct bulblet regeneration from mature embryo: a rapid, efficient and genotype-independent *in vitro* morphogenesis pathway for preservation of endangered wild populations of *Fritillaria imperialis* and *Fritillaria*

persica. HortScience, 41: 1066-1066.

MOHAMMADI-DEHCHESHMEH M., KHALIGHI A., NADERI R., SARDARI M., EBRAHIMIE E. (2008). Petal: a reliable explant for direct bulblet regeneration of endangered wild populations of *Fritillaria imperialis* L. Acta Physiologiae Plantarum, 30: 395-399.

MUCCIARELLI M., FAY M. F. (2013). Plastid DNA fingerprinting of the rare *Fritillaria moggridgei* (Liliaceae) reveals population differentiation and genetic isolation within the *Fritillaria tubiformis* complex. Phytotaxa, 9: 1-23.

MUCCIARELLI M., FERRAZZINI D., BELLETTI P. (2014). Genetic variability and population divergence in the rare *Fritillaria tubiformis* subsp. *moggridgei* Rix (Liliaceae) as revealed by RAPD analysis. Plos One, 9: e101967.

MURASHIGE T., SKOOG F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-97.

NEELAKANDAN A. K., WANG K. (2012). Recent progress in the understanding of tissue culture induced genome level changes in plants and potential applications. Plant Cell Reports, 31: 597-620.

NIKOLIĆ M., MISIĆ D., MAKSIMOVIĆ V., JEVREMOVIĆ S., TRIFUNOVIĆ M., SUBOTIĆ A. (2008). Effect of low temperature on rooting rate and carbohydrate content of *Fritillaria meleagris* bulbs formed in culture *in vitro*. Archives of Biological Sciences, Belgrade, 60: 5-6.

PAEK K. Y., MURTHY H. N. (2002). High frequency of bulblet regeneration from bulb scale sections of *Fritillaria thunbergii*. Plant Cell, Tissue Organ Culture, 68: 247-252.

PARMAKSIZ I., KHAWAR K. M. (2006). Plant regeneration by somatic embryogenesis from immature seeds of *Sternbergia candida* Mathew et T. Baytop, an endangered endemic plant of Turkey. Propagation of Ornamental Plants, 6: 128-133.

PETRIĆ M., SUBOTIĆ A., JEVREMOVIĆ S., TRUFUNOVIĆ M. (2011). Somatic embryogenesis and bulblet regeneration in snakehead fritillary (*Fritillaria meleagris* L.). African Journal of Biotechnology, 10: 16181-16188.

REED B. M., SARASAN V., KANE M., BUNN E., PENCE V. (2011). Biodiversity conservation and conservation biotechnology tools. In Vitro Cellular & Developmental Biology-Plant, 47: 1-4.

RODE C., LINDHORST K., BRAUN H. P., WINKELMANN T. (2012). From callus to embryo: a proteomic view on the development and maturation of somatic embryos in *Cyclamen persicum*. Planta, 235: 995-1011.

SANTA-CATARINA C., SILVEIRA V., BALBUENA T. S., VIANA A. M., ESTELITA M. E. M., HANDRO W., FLOH E. I. S. (2006). IAA, ABA, polyamines and free amino acids associated with zygotic embryo development of *Ocotea catharinensis*. Plant Growth Regulation, 49: 237-247.

SHARROCK S., JONES M. (2009). Conserving Europe's threatened plants: Progress towards Target 8 of the Global Strategy for Plant Conservation. Richmond, UK, Botanic Gardens Conservation International, 56 pp.

SUBOTIĆ A., TRIFUNOVIĆ M., JEVREMOVIĆ S., PETRIĆ M. (2010). Morpho-histological study of direct somatic embryogenesis in endangered species *Fritillaria meleagris*. Biologia Plantarum, 54: 592-596.

UMA S., LAKSHMI S., SARASWATHI M. S., AKBAR A., MUSTAFFA M. M. (2012). Plant regeneration through somatic embryogenesis from immature and mature zygotic embryos of *Musa acuminata* ssp *burmannica*. In Vitro Cellular & Developmental Biology-Plant, 48: 539-545.

ZHANG O., HU H., HUANG Y., HAN K., XV H., SHEN Y., LI S., HUANG J. (2012). The relationship between developmental stages of zygotic embryos at explanting and embryogenic frequency on hickory (*Carya cathayensis* Sarg.). Scientia Horticulturae, 139: 66-70.

ZOU J. J., GAO W., CAI X., ZENG X. L., WANG C. Y. (2014). Somatic embryogenesis and plant regeneration in *Osmanthus fragrans* Lour. Propagation of Ornamental Plants, 14: 32-39.