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A threatened alpine species, *Fritillaria tubiformis* subsp. *moggridgei*: Seed morphology and temperature regulation of embryo growth

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Abstract

Alpine plants have evolved to fit their life cycle into the short vegetative season of mountain habitats.*Fritillaria tubiformis* Gren. & Godr. subsp. *moggridgei* (Boiss. & Reuter ex Planch.) Rix (Liliaceae) is an endemic alpine geophyte, bearing seeds with underdeveloped embryos. Seeds are dispersed in August and embryos complete their development by spring when seeds germinate. In order to optimize seed banking procedures and to develop a proper germination protocol for plant regeneration, we studied embryo morphogenesis and analyzed how this process is influenced by temperature. Radicle protrusion occurred after an incubation of 5 months at 4°C. Under these conditions, underdeveloped embryos reached maturity and acquired a well-defined shoot apex. At the time of dispersal, abundant storage compounds were present in seeds. Lipids and lipid/proteins were uniformly distributed within the embryo and the endosperm, respectively. At late stages of embryo development, starch granules were localized at the cotyledonary tip and were also detected around the shoot meristem. Results suggested that *F. tubiformis*embryos resumed growth over a large range of temperatures, but were only able to complete development at low temperatures after which they were able to germinate by spring.

Keywords

Cold treatment, Fritillaria, seed germination, starch deposition, storage compounds, underdeveloped embryo

Introduction

The above-ground life cycle of many alpine species consists of a short vegetative period, condensed between the end of spring and summer. Seedling establishment under field conditions proceeds at a very slow rate (Bliss 1971; Forbis & Doak 2004), normally taking several years for the plant to be safely settled (Billings & Mooney 1968). In many alpine species, germination occurs as soon as soil temperatures are above freezing temperatures; any delay in this process is detrimental for radicle protrusion and plant emergence. Through adaptation to the prevailing environment, different types of dormancy and/or germination requirements have evolved such that germination occurs under favorable conditions (Korner 2003; Finch-Savage & Leubner-Metzger 2006 and references therein).

In many genera of the Liliaceae, the embryo is underdeveloped at dispersal (Martin 1946) and it must grow before germination occurs. This condition has been termed “morphological dormancy” (MD) (Nikolaeva 1977; Baskin & Baskin 2004), since radicle protrusion is delayed and germination occurs only when embryos have reached a critical length (Baskin & Baskin 2001; Baskin & Baskin 2004). The critical embryo length (CEL) is determined for seeds showing a split seed coat

and no radicle protrusion (Vandelook & Van Assche 2008); stages of embryo development are estimated by the embryo-to-seed length ratio (E:S ratio) (Forbis et al. 2002).

A number of studies have indicated that in some MD species, i.e. *Apium graveolens*, *Conium maculatum,* and *Pastinaca sativa*(Vandelook et al. 2007 and references therein), there is no additional mechanism preventing embryo growth and seed germination at optimal conditions. In contrast, in some temperate and alpine species of Liliaceae, a cold stratification treatment is necessary to break physiological dormancy (Vandelook & Van Assche 2008).

An analysis of the establishment, maintenance, and breaking of dormancy at the physiological level has shown that desiccation-sensitive and nondormant mutants of *Arabidopsis* possess impaired or decreased amounts of storage compounds (Bewley & Black1994). Storage compounds found in seeds are generally carbohydrates, lipids, and proteins (Dickie & Stuppy 2003). The first may be present either in the form of starch or as thickened cell walls of the endosperm (*Palmae* and many Liliales families), while cytoplasmic oil bodies, often in combination with protein bodies, are localized in endosperm and embryo cells (Dickie & Stuppy 2003). Desiccation-tolerance in seeds is related to the synthesis of several proteins, which are involved, together with lipids, in the establishment of desiccation-tolerant cell membranes, seed drying (Soeda et al. 2005), and in protection and repair mechanisms activated before and during seed germination (Bewley & Oliver 1992).

All main classes of plant hormones are thought to be involved in dormancy release through different effects on seed physiology and also by the activation of catabolic pathways leading to seed storage mobilization (Kucera et al. 2005 and references therein). Furthermore, mobilization rates of stored lipids can affect the germination process itself, its velocity, and how the process is regulated by temperature (Lehner et al. 2006).

According to the literature, five *Fritillaria* species have underdeveloped embryos and physiological dormant seeds – morpho-physiological dormancy (MPD) (*sensu* Nikolaeva 1977; Finch-Savage & Leubner-Metzger 2006; Kondo et al. 2006). Nevertheless, literature data on temperature requirements for embryo development and germination in *Fritillaria* are lacking.

Considering the importance of establishing suitable propagation techniques for threatened species, we analyzed embryo development of *Fritillaria tubiformis* Gren. & Godr. subsp. *moggridgei* (Boiss. & Reuter ex Planch.) Rix (Liliaceae), and how this process is regulated by temperature up to germination. We also located lipids, proteins, and starch granules within the seeds at dispersal and starch distribution during embryo development. *F. tubiformis* was studied because it is a rare endemic species growing in the Ligurian and Maritime Alps (NW Italy) (Aeschimann et al. 2004; Conti et al. 2005) between 800 and 2100 m a.s.l. (Pignatti 1982). The life cycle of this species is characterized by a relatively short vegetative growth, which begins in early spring and relies on rapid seedling emergence from soil at the snow melt period (personal observations).

Materials and methods

Plant material

Seeds were collected on 4 August 2006 in the Alta Valle Pesio e Tanaro Natural Park (Piedmont, Italy) from a population of *F. tubiformis* subsp. *moggridgei* growing at Pian del Lupo (1999 m a.s.l.). Only dry fruits containing ripe seeds were harvested. After 1 week under ambient conditions, seeds were dried to equilibrium in a drying room (15°C and 15% RH) and then stored at −20°C.

Growth conditions, morphometry, and seed germination

To start germination tests, seeds were thawed and equilibrated to room temperature for 1 week. Six replicates of 25 seeds (150 seeds) were sown on agar (1% w/v in water) in 9-cm glass Petri dishes and incubated in the dark, at each of the following constant temperatures: 4, 10, and 20°C. Further six replicates were cycled through a seasonal temperature regime of 30 days at 5/10°C (autumn), 60 days at 4°C (winter), 30 days at 5/10°C (spring), 30 days at 10/20°C (summer). During incubation, every 30 days up to 150 days, 10 seeds were randomly selected from each temperature regime, and used for both morphometric and histological analyses. Germination percentages were calculated after 150 days on the remaining 100 seeds.

Due to seed coat transparency, embryo morphometry was carried out by trans-illuminating seeds under a stereoscope equipped with a CCD video camera (Nikon DS-Fi1). Images were acquired with Photoshop 7.0 (Adobe Systems Inc.). Embryo and seed lengths were measured with *Image-Pro Plus* version *6.2 software*. Seed length corresponded to the distance between the micropylar and the extreme chalazal ends, excluding the peripheral wing of the seed. Embryo length was measured from the radicle to the cotyledonary tip, and the embryo-to-seed ratio (E:S) was calculated for each seed.

Microscopy

Seeds were dissected into the two main components (embryo and endosperm). Hand sections of the endosperm were stained with Calcofluor White ST (Sigma-Aldrich) fluorescent brightener (0.5% w/v in water) (DeMason 1986) to detect cellulose and other polysaccharides (Hughes and McCully 1975). Sections were observed with an epifluorescence microscope (Nikon Eclipse E600 UV-2A) equipped with a blue/UV emission filter (420 nm) at an excitation of 330/380 nm.

Embryos were extracted from seeds and fixed with a 1.5% (v/v) glutaraldehyde/formalin (1:1) mixture in 0.05 M phosphate buffer (pH 7.5) for 3 h at 4°C (modified from Hall & Hawes 1991). They were rinsed twice with phosphate buffer for 5 min and post-fixed in OsO_4 1% (v/v) for 2 h (Panza et al. 2004; Xie et al. 2005). Embryos were rinsed, dehydrated through a graded ethanol series, and embedded in LR White resin (Sigma) at 60°C for 2 days.

Semi-thin sections (1-µm thick) were cut with glass knives on a microtome (Reichert-Jung Ultracut E) and mounted on glass slides by gently heating at 50°C on a slide warmer. Sections were stained with toluidine blue 0.05% (w/v), to observe the general embryo structure, and with periodic acid-Schiff's reagent (Merck) (PAS reaction), to trace insoluble carbohydrates (Cadot et al. 2006). Starch distribution was observed on transversal sections of germinating embryos (150 days incubation) stained with toluidine blue and PAS reagent.

Both fresh freehand and semi-thin sections were used to detect lipids and proteins in seeds at dispersal. Lipids were stained with Nile red (Fluka) (0.01% w/v in acetic acid), and proteins with naphthol blue black (Fluka) (1% w/v in 7% acetic acid water solution) (Clark 1981).

Images were acquired through conventional microscopy by a CCD video camera (Nikon DS-Fi1) with NIS-Elements F 2.30 software.

Statistics

Lengths and percentages are expressed as means ± SE, when appropriate, and were compared by analysis of variance (ANOVA) using Systat 11 software for Windows. Differences among treatments were considered statistically significant at $p < 0.05$ (Tukey–Kramer post-hoc test). Exponential germination curves were fitted to the embryo seed data in Genstat 11 (VSNi Ltd., UK) with temperature as a treatment factor.

Results

The principal features of the seed and embryo of *F. tubiformis* subsp. *moggridgei* are shown in Figure 1. The average length ± SE of the seed at dispersal is 5.7 ± 0.2 mm. As a main feature of the genus, the seed is ovate, almost triangular in shape with a narrowing tip at the micropylar region. The seed is flattened, and has a wing-margined contour. Seed surface is cream-brown in color, and evenly reticulated as a consequence of the irregular shape and undulate margins of testa cells (Khaniki 2003).

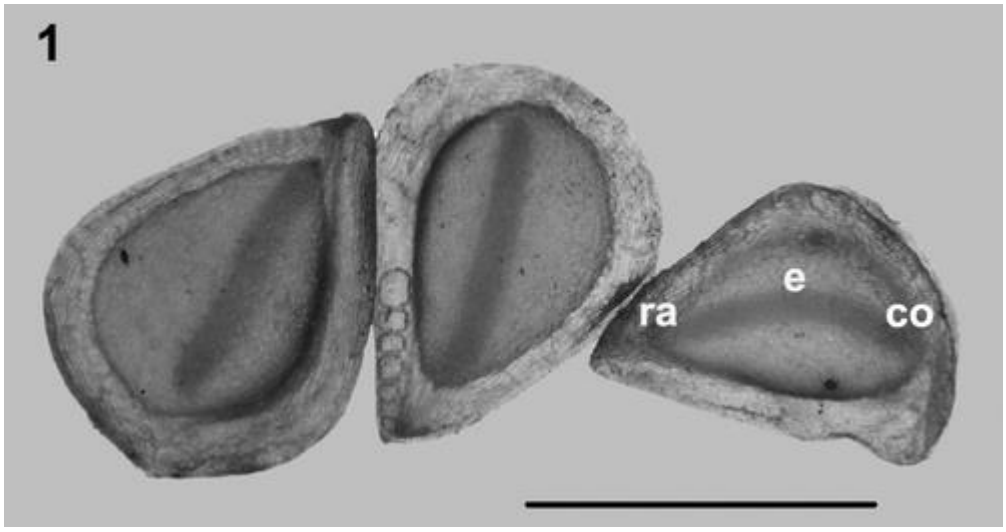
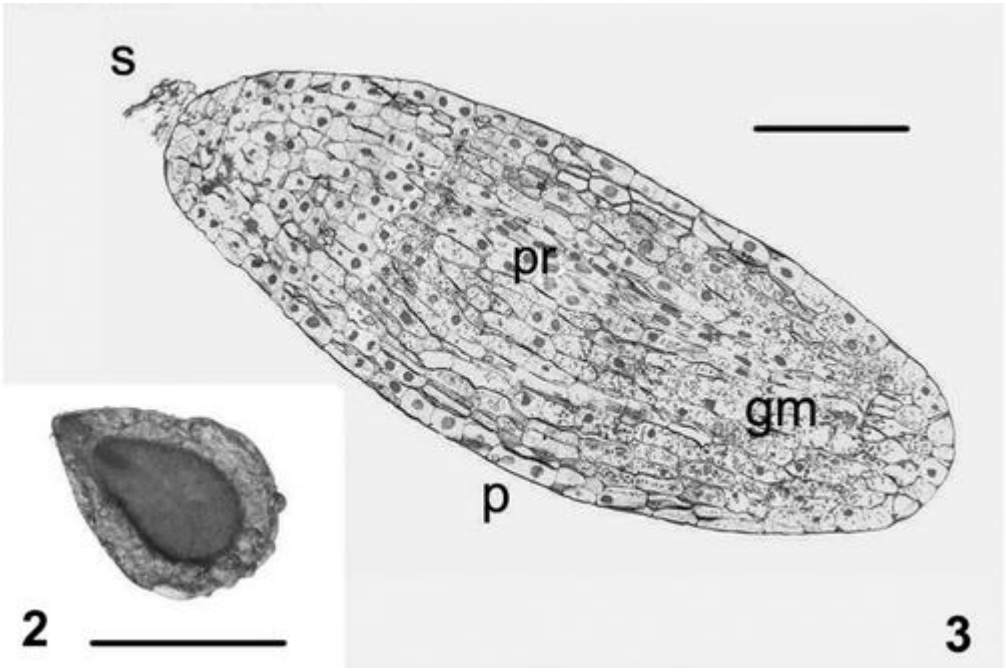
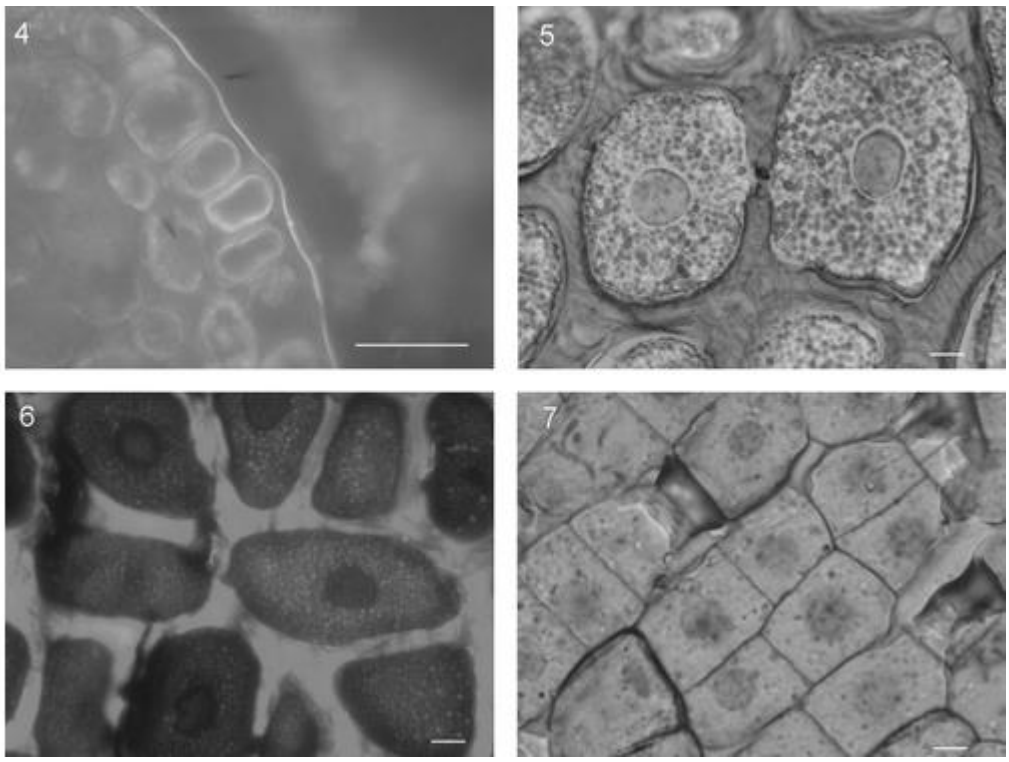


Figure 1. Seeds of *F. tubiformis* subsp. *moggridgei* with a fully developed linear axil embryo. Bar = 5 mm. e, embryo; ra, root apex; co, cotyledon tip.

At dispersal, the embryo was very small (Figure 2) with an average length of 0.83 ± 0.02 mm, corresponding to 14.7% of the seed length. The embryo is elongated with the root pole recognizable by the remains of the suspensor (Figure 3); it can be regarded as underdeveloped *sensu* Baskin and Baskin (2004).



Figures 2–3. Seed and embryo of *F. tubiformis* subsp. *moggridgei* at the time of dispersal (no incubation). (2) A seed at dispersal containing a minute embryo (E:S ratio 14.7%). Bar = 5 mm. (3) The underdeveloped embryo in longitudinal section. Bar = 100 µm. s, suspensor; p, protoderm; pr, procambium; gm, ground meristem.



Figures 4–7. Storage compounds (proteins and lipids) in seeds of *F. tubiformis* subsp. *moggridgei*. (4) Nile red fluorescent brightener stains lipids in embryo cells. A fluorescent cuticle enclosing the embryo is also evident. Bar = 50 µm. (5) Abundant protein bodies filling the endosperm cells of a different seed at dispersal (naphthol blue black staining). Bar = 10 µm. (6) Copious oil bodies are visible in the endosperm of another seed of the same age (Nile red fluorescent brightener). Bar = 10 µm. (7) Protein bodies are scarce in the young underdeveloped embryo. Bar = 10 µm.

In the embryo, a layer of protodermal cells was present surrounded by a thin cuticle when observed under fluorescence (Figure 4). The embryo was embedded in a moderately hard endosperm, with thickened cell walls. Endosperm cells contained abundant proteins (Figure 5) and oil bodies throughout the cytoplasm (Figure 6). At dispersal, storage substances in the embryo were represented by cytoplasmic oil bodies (Figure 4), and by few proteins bodies (Figures 7 and 8).

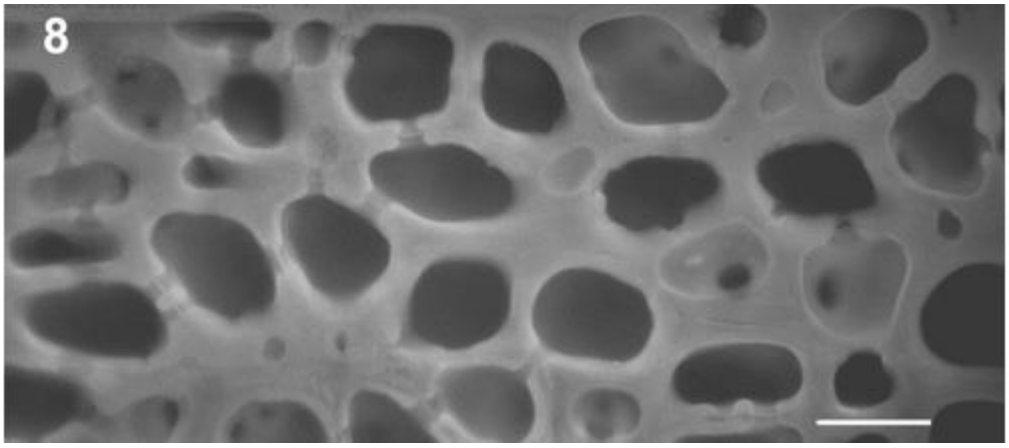


Figure 8. Hand-cut section of the thick-walled endosperm of *F. tubiformis* subsp.*moggridgei* seed (Calcofluor White ST fluorescent brightener). Bar = 100 µm.

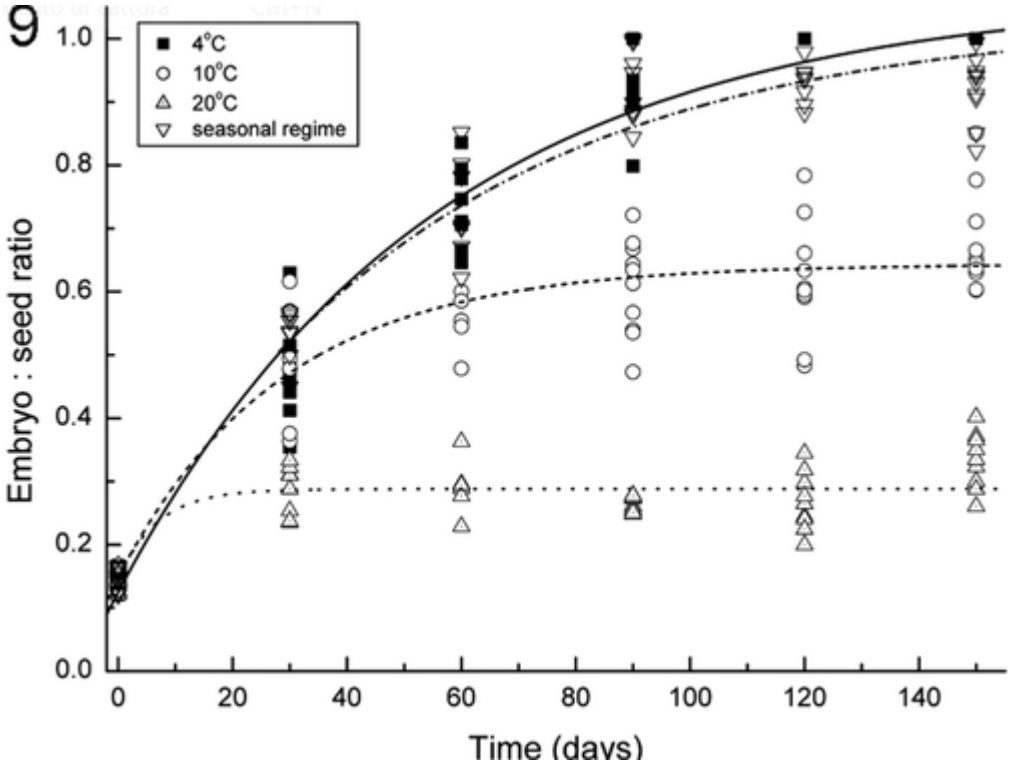
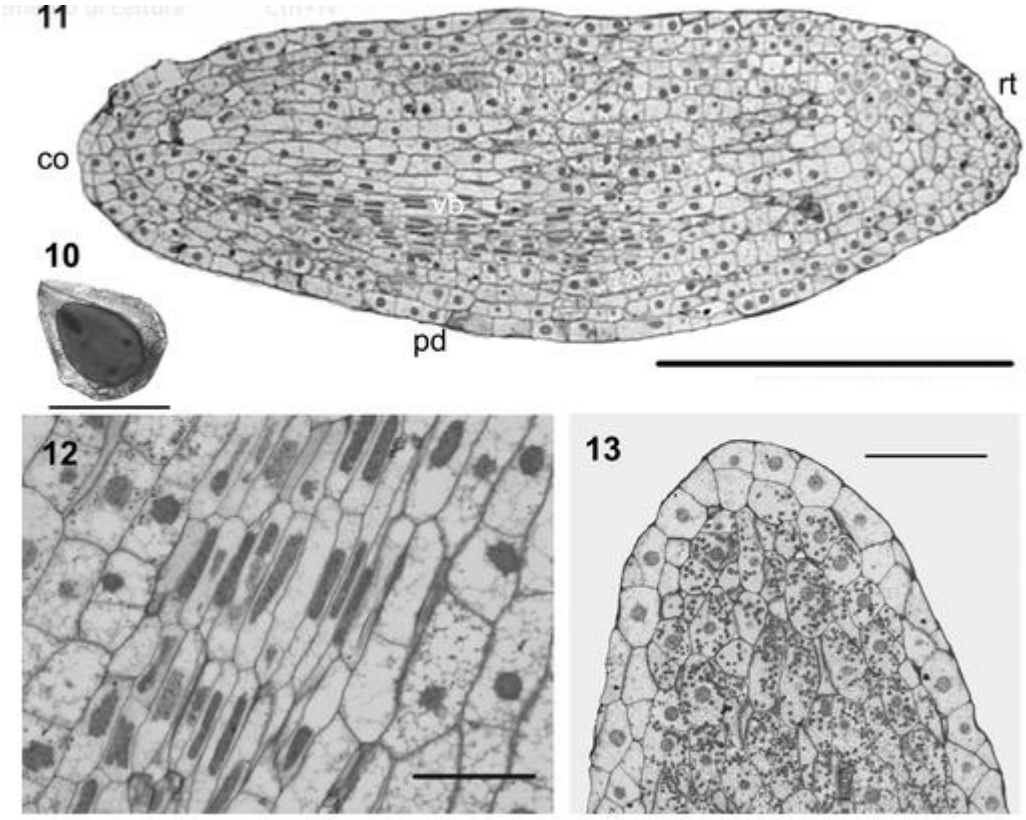
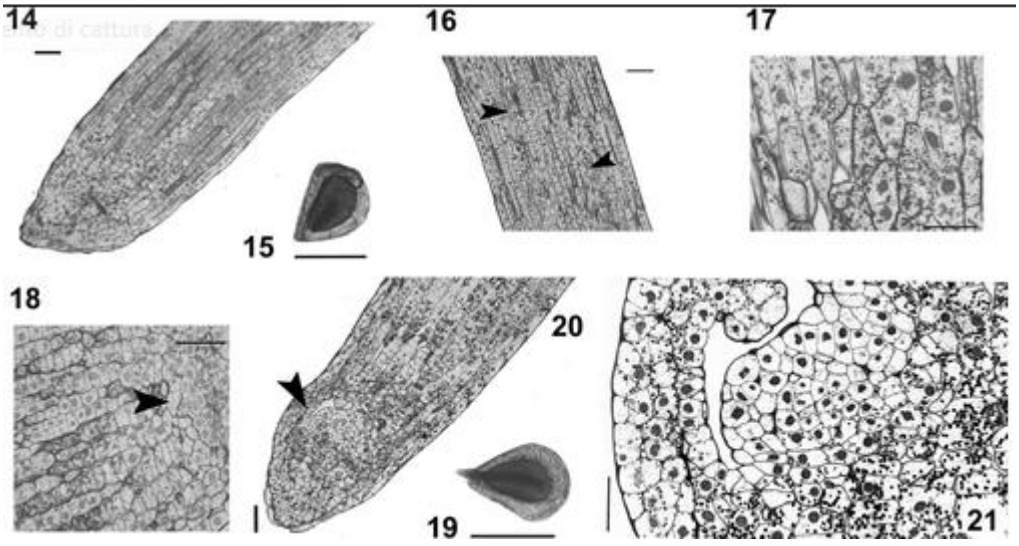


Figure 9. Embryo growth in seeds of *F. tubiformis* subsp. *moggridgei* incubated at different temperatures. Embryo length is expressed as E:S ratio and modeled using exponential curve fitting. For seeds placed under different temperatures (4, 10, and 20°C; solid square, open circle, and open up-triangle, respectively) or the seasonal regime (open down-triangle), the curves were significantly different from each other (p<0.05).

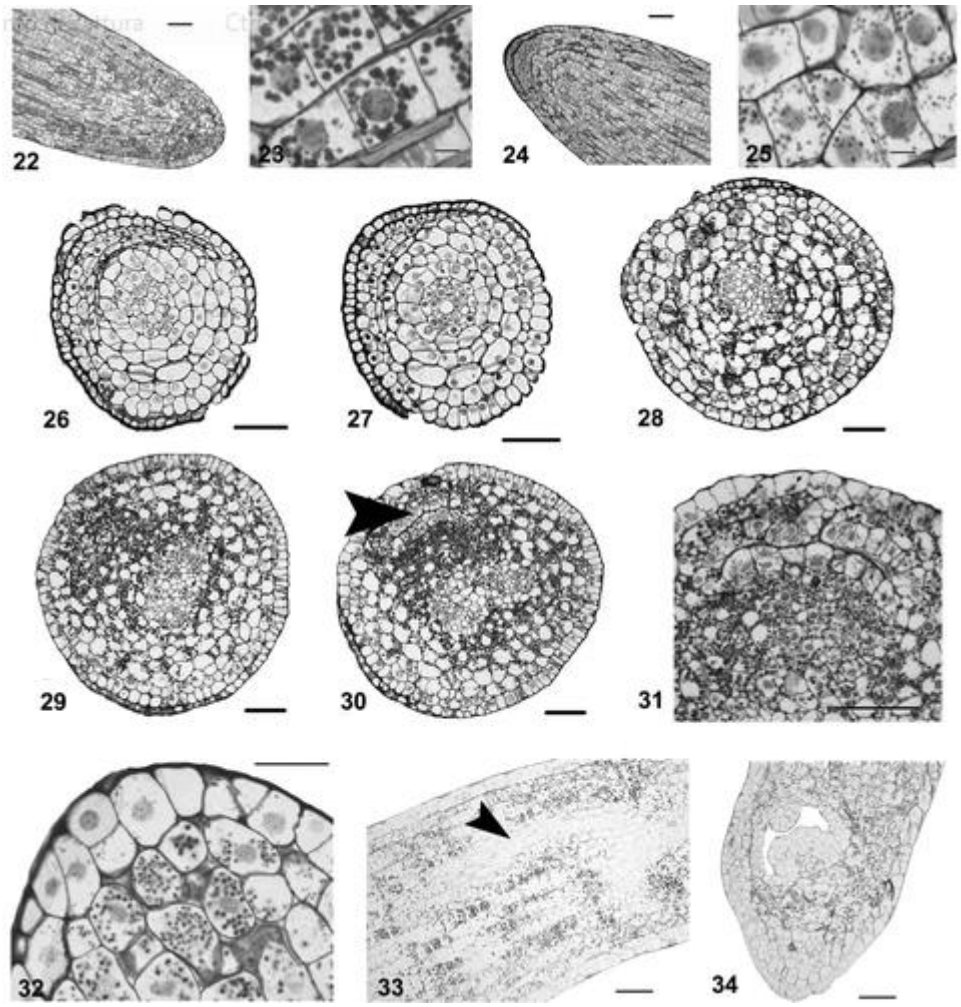


Figures 10–13. Seeds and embryos of *F. tubiformis* subsp. *moggridgei* after 150 days at 20°C. (10) Embryo size within the seed is almost unchanged. Bar = 5 mm. (11) Embryo longitudinal section showing protoderm (pd), procambium (vb), and the meristematic root (rt). Bar = 500 µm. co, cotyledon tip. (12) Long narrow cells and elongated nuclei characterize the longitudinal procambium which lies in the middle portion of the embryo. Bar = 50 µm. (13) Numerous large starch grains are present at the cotyledonary tip; the protoderm is devoid of starch. Bar = 100 µm.



Figures 14–21. Seeds and embryos of *F. tubiformis* subsp. *moggridgei* after 150 days at 10°C (14–18) and at 4°C (19–21). (14) The root apex of the embryo is well distinguished. Bar = 100 µm. (15) A 150–day-old seed incubated at 10°C. It bears a more elongated embryo compared with the seeds at 20°C (E:S ratio 67.7%). Bar = 5 mm. (16) Longitudinal sections of the embryo showing two developing vascular bundles (arrows) lying in the middle portion of the embryo. Bar = 100 µm. (17) Starch grains at the cotyledon tip. Bar = 50 µm. (18) The future meristematic shoot consists of a

clump of dividing cells (arrow) positioned at the bottom of the cotyledonary axis and just above the root apex (here on the right, not fully shown). Bar = 100 µm. (19) At the end of incubation at 4°C, the root apex protrudes through the seed coat. Bar = 5 mm. (20). The SAM arises as a lateral protuberance in the basal part of the embryo (arrow). Bar = 100 µm. (21) The figure shows a detail of the SAM, protruding from one side of the embryo and consisting of dividing cells arranged in circular rows. Bar = 500 µm.



Figures 22–34. Starch deposition in 60-day-old (22–25) and 150-day-old embryos (26–34) incubated at 4°C. (22) Strong deposition of starch in the cotyledon tip. Bar = 100 µm. (23) Detail of cotyledonary starch grains (Ø 3–5 µm). Bar = 10 µm. (24) Starch deposition at the root apex. Bar = 100 µm. (25) Detail of the starch grains (Ø 0.5–2 µm) at the root apex. Bar = 10 µm. (26–31) Transversal sections of a germinated embryo of *F. tubiformis* subsp. *moggridgei* showing the main patterns of starch deposition between root and shoot meristems. See text for a detailed description. Arrow in Figure 30 indicates the lateral sheath of cells protecting the future shoot apex. Bar = 100 µm. (32–34) Deposition of starch in longitudinal sections of the embryo after 150 days at 4°C. (32) The cotyledon tip is shown. The protoderm is devoid of starch. Bar = 50 µm. (33) Starch reserves are abundant in parenchymatic cells of the middle-basal embryo portion and excluded from the protoderm, the procambium (arrow), and the meristematic area. Bar = 100 µm. (34) The SAM is free of starch grains, and lies enclosed in a curved cell pocket.

Embryo growth rate varied according to the temperature (Figure 9). Seeds incubated at constant 20°C showed a slight E:S increase during the first 30 days of incubation, before reaching a mean embryo length of 1.45 ± 0.05 mm by the end of the experiment (150 days) (Figure 9, Table I). Seeds did not germinate. On the other hand, at 10°C, 4°C, and under the seasonal regime, the E:S ratio increased more than three-fold within the first 30 days of incubation. Differences between treatments were not significant ($p > 0.05$; Figure 9, Table I). Under these conditions, E:S ratio values continued to increase up to the end of the experiment, but with different patterns.

Table I. Mean embryo length (mean ± SE) of *F. tubiformis* subsp. *moggridgei* seeds, after 30, 60, 90, 120, and 150 days of incubation at different temperatures (see text for details)

Treatments	30	60	90	120	150	%
+4°C	1.86 ± 0.12b	2.93 ± 0.12c	4.55 ± 0.37d	*	*	72.8e
+10°C	2.09 ± 0.16b	2.23 ± 0.08b	2.56 ± 0.11bc	2.56 ± 0.16bc	2.88 ± 0.11c	0.0f
+20°C	1.19 ± 0.06a	1.23 ± 0.05a	1.10 ± 0.03a	1.64 ± 0.35ab	1.45 ± 0.05a	0.0f
Seasonal	2.24 ± 0.05b	3.04 ± 0.06c	3.86 ± 0.10d	4.09 ± 0.13d	3.97 ± 0.08d	19.3f
Notes: Embryo length was 0.83 ± 0.02 mm at the start of the experiment, and increased significantly in all treatments after an incubation of 30 days. In the last column, final germination (%) at 150 days of incubation has been also reported.						
Different letters indicate significant differences among data ($p < 0.05$).						
*Embryo length is not reported because most seeds were already germinated.						

Table I. Mean embryo length (mean ± SE) of *F. tubiformis* subsp.*moggridgei* seeds, after 30, 60, 90, 120, and 150 days of incubation at different temperatures (see text for details)

At the end of the experiment at 10°C, the maximum embryo length was 2.88 ± 0.11 mm (Table I), and the final E:S value was about double that of seeds incubated at 20°C (Figure 9, Table I), however, no germination occurred under this treatment. At 4°C and with the seasonal regime, the E:S ratio increased sharply up to day 90 (to about three times the E:S ratio of embryos at 20°C) (Figure 9). After 150 days at the seasonal regime, embryos were on an average 3.97 ± 0.08 mm long (Table I) but they did not reach the CEL (92.1% E:S ratio; Figure 9). The final germination percentage was only 19.3%. On the other hand, embryos kept at constant 4°C reached the critical length for germination within 120 days of incubation (E:S ratio 100%) (Figure 9) and germinated (72.8%).

Consistent with the increase in embryo size, the overall embryo morphology changed with time and according to the temperature of incubation.

When embryos were held at 20°C, their size was slightly increased by the end of the experiment (Figure 10) but they were still underdeveloped. Figure 11 exemplifies the longitudinal section of this embryo and shows that both leaf and root tips were hardly recognizable after 150 days of incubation. The shoot apex was absent. Long and narrow procambial cells with elongated nuclei occurred in the middle cotyledonary portion of the embryo (Figure 12). A clump of isodiametric meristematic cells, possibly the precursor of the shoot meristem, was evident in some embryos a short distance (about 100 µm) from the root apex. Large starch granules were concentrated at the cotyledonary tip (Figure 13), whilst they were scarce and small at the opposite pole, around the procambium, and almost excluded from the protoderm.

Incubation for 150 days at 10°C resulted in a considerably elongated and developed embryo (E:S ratio 67.7%) (Figures 14 and15). The cotyledon was cylindrical in the proximal portion, often flattened at the distal end. It consisted of actively dividing cells arranged in longitudinal rows and contained two longitudinal procambial bundles (Figure 16 arrows). Cells of the cotyledonary tip were full of starch grains (Figure 17). Starch grains were also present in the remaining part of the cotyledon, though smaller and less frequent. The shoot apical meristem (SAM) was situated at the cotyledon base and consisted of polyhedral cells almost devoid of starch granules (Figure 18 arrow).

After 150 days at 4°C, embryos had completed their development and extended throughout the endosperm, and their root apices protruded through the seed coat (Figure 19). The morphogenetic level reached by the embryos incubated at 4°C is exemplified by Figures 20 and 21.

Embryos showed an evident SAM and a well-developed cotyledon bearing two procambial bundles extending toward the base of the apical meristem, where they joined together. The SAM appears like a lateral protuberance enclosed within a curved cell pocket (Figures 20 and 21). Transversal sections of the root showed a large differentiating metaxylem vessel at the centre of the future stele, as generally occurs in Liliaceae (Tonzig & Marrè 1976; Fahn 1990), with the cortical precursor cells arranged in concentric layers and surrounded by a protoderm (Figures 26 and 27). Embryos of seeds incubated at the seasonal regime showed histological features comparable with those incubated at 4°C, although they did not reach the critical length (not shown).

Starch reserves were very scarce in the embryo at dispersal, increased during embryo growth, and became reorganized following a pattern related to embryo development. In embryos incubated at 4°C, starch reserves were scarce up to 30 days of incubation. From 60 days onward, large starch grains (3–5 μm) become widespread in all the cotyledonary cells (Figures 22 and 23), except for the protoderm. At the root apex, grains were small (0.5–2 μm in diameter) and less numerous (Figures 24 and 25). With time, grain concentration decreased at the cotyledonary tip and increased in the cells surrounding the procambial bundles and the SAM.

Transversal sections of 150 days embryos helped to clarify the distribution of the starch granules within the root apex and the cotyledon (Figures 26–31). Starch was absent in the root (Figures 26 and 27) and appeared in the differentiating cortical cells of the hypocotyl zone (Figure 28). Starch concentration increased further toward the shoot apex, and the highest amount was found around the dividing cells giving rise to the SAM (Figures 29–31). In longitudinal sections of a mature embryo (150 days of incubation), PAS reaction showed that starch grains decreased at the cotyledonary tip (Figure 32) and became concentrated in the embryo zone, particularly in the ground tissue around the procambium and the dividing cells of the shoot apex, whilst they were excluded from the procambium, the protoderm, and the SAM (Figures 33 and 34).

Discussion

Seeds of *F. tubiformis* subsp. *moggridgei* (Liliaceae) resembled those of the other members of the genus *Fritillaria* studied to date (Khaniki 2003). They showed underdeveloped embryos *sensu* Baskin and Baskin (2004). At dispersal, the embryo occupied a small portion of the seed (average E:S ratio 14.7%). This condition represents a morphological restriction to seed germination because embryo development must be completed before germination can take place (Stokes 1952).

Embryos which need additional growth after dispersal have been found in several families of dicotyledons and monocotyledons, in tropical and temperate regions, e.g. Magnoliaceae,

Annonaceae, Ranunculaceae, Papaveraceae, Apiaceae, Araliaceae, and Liliaceae (Werker 1997 and references therein; Baskin & Baskin 2001). Underdeveloped embryos have also been documented in a few species of the alpine habitats (Forbis & Diggle 2001), where germination timing is pivotal to allow plant establishment in these harsh environments (Forbis et al. 2002).

Our results showed that temperature determined the time of germination in *F. tubiformis* by controlling the rate of embryo growth. A prolonged cold stratification prompted a timely and uniform embryo growth and, in fact, it was necessary for a full embryo development. Embryos reached the critical length after 120–150 days at 4°C, and by the end of the experiment (150 days), they germinated. On the contrary, only a few seeds germinated at the seasonal regime since embryos had not yet reached the critical size (mean E:S 92.1%). In fact, the sudden increase in temperature (from 4 to 5/10°C) experienced by embryos did not affect the final embryo morphology, but strongly delayed radicle protrusion. After 150 days of incubation, embryos of seeds incubated at 10 and 20°C were still largely underdeveloped and they did not germinate. Thus, *in situ*, embryo growth will occur at a steady rate over winter, and embryos will reach full size and be ready for germination, when temperatures increase. This corresponds to the months of March–April at 2000 m a.s.l. (personal observation). This behavior is common in alpine environments, where, due to the short growth period, seedling development must start in the subnivean environment as soon as temperatures are above freezing (Billings & Mooney 1968; Forbis & Diggle 2001).

Low temperatures are likely to promote the cell cycle resumption of the embryo and this may be related to the activity of gibberellins (GAs). These plant hormones, in fact, are involved in the regulation of the cell cycle, and their *de novo* biosynthesis in *Arabidopsis thaliana* is stimulated by low temperatures (Yamauchi et al. 2004). The synergistic interaction of light and low temperatures has been demonstrated to promote dormancy release and germination through the transcriptional induction of the gibberellin biosynthetic gene GA_3 oxidase (Penfield et al. 2005). The effect of exogenous GA_3 on *F. tubiformis* seeds is under study.

The development of *F. tubiformis* embryos at low temperatures basically implied polarized growth and tissue specification. This process resulted in the establishment of root and shoot meristems, which were paralleled by the development of the procambium and the accumulation of intracellular storage compounds.

Our study showed that only seeds that had experienced a continuous cold period accomplished the aforementioned morphogenetic steps. This process culminated in the formation of the SAM. As in the general model of grass embryo development (Clowes1978), the apical meristem of *F. tubiformis* arose as a lateral embryo protuberance. However, different from other monocots, e.g. *Zea mays*, the coleoptile was absent, while, as in *Allium cepa*, the apical shoot was localized beneath the protective, sheath-like base of the cotyledon (Esau 1977). This developmental pathway finds support also in an article by Blodgett (1910) on*Erythronium americanum* (Liliaceae). In that species, the SAM and the leaf scale are undistinguishable at early stages of development since they are in close contact with each other and both are actively proliferating. By the time of SAM emergence, however, leaf scale margins enlarge and project forward thus completely enclosing the apical meristem (Blodgett 1910). A similar condition can be envisaged in *F. tubiformis* when observing the localization of the shoot meristem, still enclosed within this protective tissue sheath on the side of the embryo (Figures 20, 21, 30, 31, and 34).

The transition from embryo dormancy to germination involves the activation of developmental processes and the rapid mobilization of seed storage reserves; this will ultimately result in radicle

emergence, seedling establishment, and subsequent photoautotrophic growth (Bewley 1997). Knowing which main storage compounds are present within the seeds helps to understand the main mechanisms regulating seed persistence, intraseminal embryo growth, germination, and seedling establishment.

At the time of seed dispersal, proteins and lipids were the most abundant storage reserves in the endosperm of *F. tubiformis*seeds while, according to our results, the young underdeveloped embryo contained abundant lipids and a few starch grains. Lipids are energy compounds very suitable in sustaining rapid growth of embryos and when net production is reduced, as experienced by many alpine species at the time of spring emergence (Dafni et al. 1981). Storage lipid mobilization has been studied in apple seeds during cold stratification. In this species, lipases that are involved in lipid mobilization are activated by low temperatures and operate at an optimum temperature of 4°C (Smolenska & Lewak 1974; Zarska-Maciejewska & Lewak 1976). All these observations address the importance of lipids as the primary source of energy exploitable by *F. tubiformis* embryos, and their possible involvement in growth activation upon embryo imbibition and cold stratification.

The presence of oils and lipids in seeds has also important practical implications in long-term germplasm conservation. The finding that the underdeveloped embryos of *F. tubiformis* are rich in lipids suggests that the optimal moisture content of this species should be experimentally determined before proceeding with drying in order to increase seed longevity during long-term storage.

Our results showed that the quiescent embryo of *F. tubiformis* contained only small quantities of starch and none was present in the endosperm; hence, the main polysaccharides at the time of seed dispersal were those structurally incorporated within cell walls (Figure 8). After growth resumption, nevertheless, starch was detected in the embryo, and its concentration increased along with cold incubation. Only later, at the mid phase of embryo development (90 days of incubation), starch grains appeared throughout the embryo axis.

In *F. tubiformis,* a cotyledon with haustorial properties, similar to that found in other members of the Liliaceae and Alliaceae families, where embryo axial growth is supported by the degradation of the endosperm cell walls (Williams et al. 2001; Marshall & Grace 2008; Niu et al. 2009), remains to be demonstrated, also considering the large amounts of protein and lipid storages found in the endosperm cells.

Present results also showed that a secondary site of starch accumulation in *F. tubiformis* embryos was represented by the area around the developing shoot meristem. In germinating seeds, starch concentration decreased in the cotyledon but was still high all around the procambium and the shoot apex. Degradation of starch into soluble sugars is fundamental to support seedling growth since the energy threshold is high in this process. The degrading activity of enzymes, such as α- and β-amylases, limit dextrinase (debranching enzyme) and α-glucosidase during germination of cereal grains has been well documented (Godbole et al. 2004; Zeeman et al. 2007). At the time of radicle protrusion, however, starch grains were still abundant around the future SAM of *F. tubiformis* embryos. We, therefore, hypothesize that starch mobilization occurs from the cotyledon toward the shoot apex. Starch could accumulate at the shoot apex in order to support the rapid growth, after germination, of the bulb; the latter organ was already recognizable in 3–4 months-old *Fritillaria* seedlings (personal observation).

In conclusion, this work sheds light on some relevant aspects of the seed morphology of *F. tubiformis*, describing the main steps of embryo growth and morphogenesis. We have also determined the temperature regimes necessary to initiate and sustain embryo development up to germination. These data are useful for implementing germination protocols and seed banking procedures suitable for the long-term conservation of this endangered species.

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