“Support for the definition of a protocol to obtain embryogenic lines of *Abies nebrodensis* for the establishment of cryo-banks – Action A1 of the LIFE18 project NAT/IT/000164 LIFE4FIR”

Report of a complete protocol for *A. nebrodensis* somatic embryogenesis

Authors

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Table of contents:

1. Introduction

The Sicilian fir (*Abies nebrodensis* Mattei) stands as the rarest endemic conifer in the Madonie Regional Natural Park, located in the mountainous region of Polizzi Generosa in north-central Sicily, Italy. By 1900, its popularity had brought it to the brink of extinction. However, in 1957, a rediscovery a few kilometers from Polizzi Generosa revealed a small population. Various challenges, including limited embryos in seeds, low germination rates, and the threat of hybridization with non-native firs, contributed to the difficulty in propagating this species, resulting in significant genetic pollution.

The combination of strong genetic erosion and poor natural regeneration has dwindled the remaining population to a mere thirty mature trees, earning it a classification as a critically endangered species by the International Union for Conservation of Nature (IUCN). Conservation efforts, dating back to the 1940s and including successful initiatives like the EU LIFE four-year 'LIFE-Natura 2000' funded project (2001-2005), have aimed at preserving the Sicilian fir in its natural and controlled environments.

Despite these endeavors, the precarious status of the Sicilian fir necessitated further protection through innovative methods. Hence, the current EU LIFE-funded project, LIFE4FIR (2019-2023), titled 'Decisive *in situ* and *ex situ* conservation strategies to secure the critically endangered Sicilian fir, *Abies nebrodensis* - LIFE 18 NAT/IT/164 LIFE4FIR,' is dedicated to saving and sustaining this invaluable species.

2. An overview about somatic embryogenesis

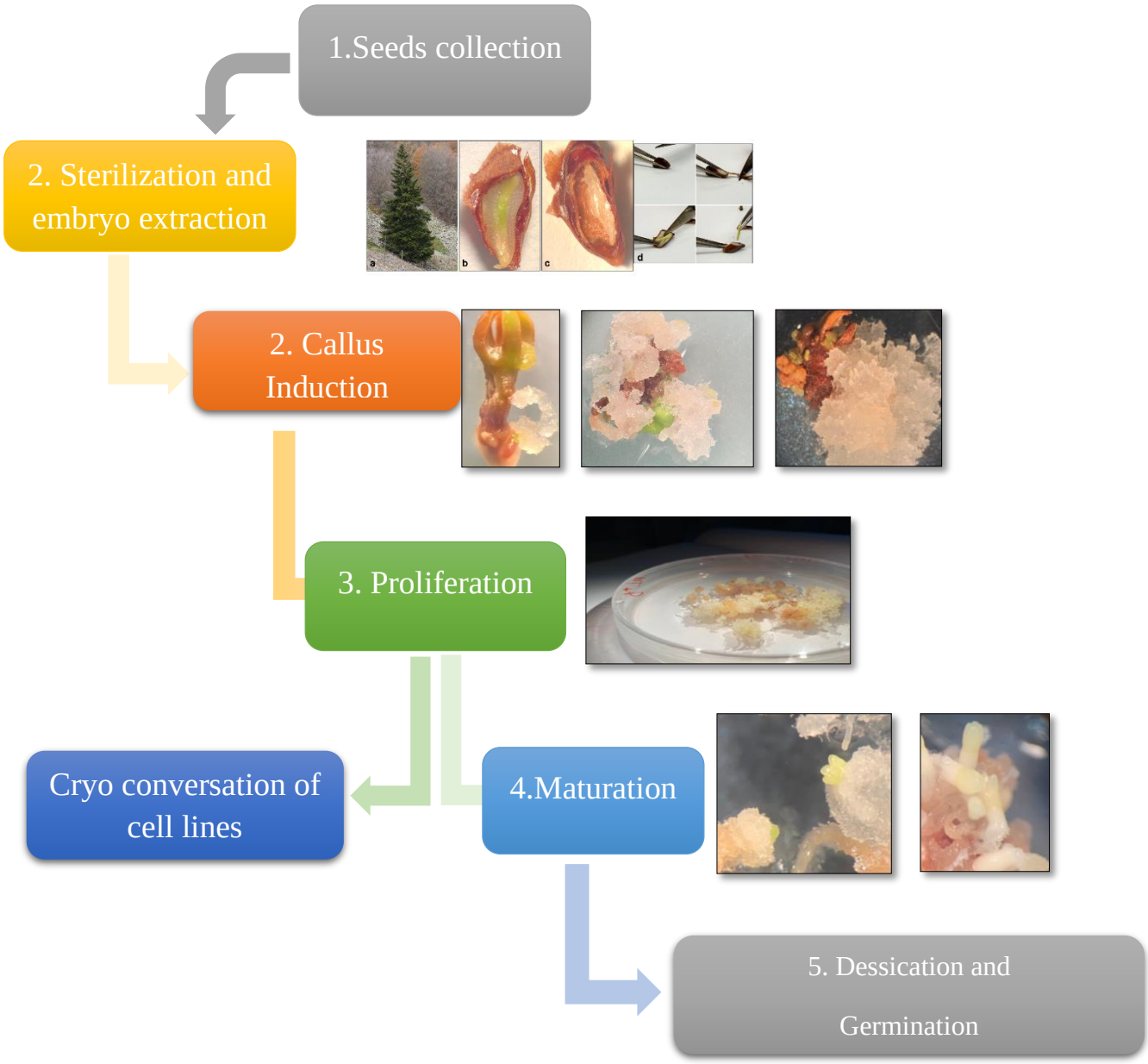
Somatic embryogenesis, recognized as an advanced tool in forestry, has been applied for over three decades, initially reported for coniferous species. The pioneering application with Norway spruce demonstrated its potential as a micropropagation approach (Hakman et al., 1985), evolving into a beneficial method for ecologically and economically significant forestry species. Ongoing research on Norway spruce (*Picea abies* L. Karst.) and Scots pine (*Pinus sylvestris* L.) has delved into understanding the mechanisms governing tree embryogenesis and in vitro cultivation (Hazubska-Przybył et al., 2022).

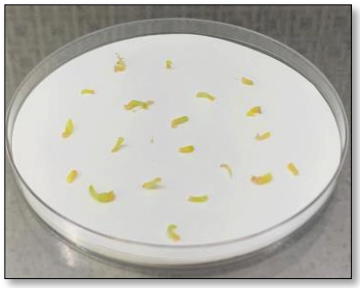
For the genus Abies, somatic embryogenesis was introduced by Schuller et al. in 1989, and this technique has since been applied to various fir species, such as *Abies alba* × *Abies nordmanniana* and *Abies alba* × *Abies cephalonica* (Vookovà et al., 1997; Salajová et al., 1996; Salajová and Salaj, 2003). Combining this approach with cryoconservation of embryogenic

tissue enables large-scale propagation and preservation of forestry resources (Lelu-Walter et al., 2013). Substantial success has been achieved in producing emblings from somatic embryos of commercially important species, with large-scale field trials established (Loyola and Ochoa, 2016; Isah, 2016). However, for *Abies nebrodensis*, obtaining embryogenic callus has proven elusive (Krajňáková et al., 2014).

The primary aim of this paper is to establish highly reproducible *in vitro* approaches, specifically focusing on somatic embryogenesis and encapsulation technology. Notably, the study presents the first-ever protocol for somatic embryogenesis derived from mature zygotic embryos of *Abies nebrodensis*.

3. Steps to establish the protocol for somatic embryogenesis





3.1. Plant material: donor trees and seed collection

Figure 1. Mature adult *Abies nebrodensis* Tree, located in the Madonie park

Individuals selected in the present study are the critically endangered adult trees of *Abies nebrodensis* (Figure1*)*, endemic to the north-central part of Sicily. The population is limited to thirty residual individuals, distributed discontinuously in around 100 hectares in the Madonie park, in the 'Vallone Madonna degli Angeli' (approximate location 37,85°N, 14,04°E), Polizzi Generosa (Sicily). Cones were collected and stored dry at 4 °C before culture initiation.

Immature and mature cones were harvested, after open-pollination. Immature cones containing seeds at late embryogeny stage, were collected at two different dates mid- and late-July 2020, while mature cones were harvested during the last week of September 2020.

3.2. Selection of full seeds

Two replicates of 50 seeds from each tree were radiographed, with the aid of digital equipment (Gilardoni radio light, Lecco, Italy) connected to a computer. The X-ray plates were evaluated based on both embryo presence and morphology. Seeds previously subjected to the X-Ray imaging test were checked on X-Ray Viewer Screen (Figure 2). Consequently, seed was considered abnormal if one or both structures (embryo cavity and endosperm) were deformed. Empty seeds were separated from the full ones and verified by opening the seed sample under the stereoscope (Figure 3). The percentage of seeds with or without embryos was determined.

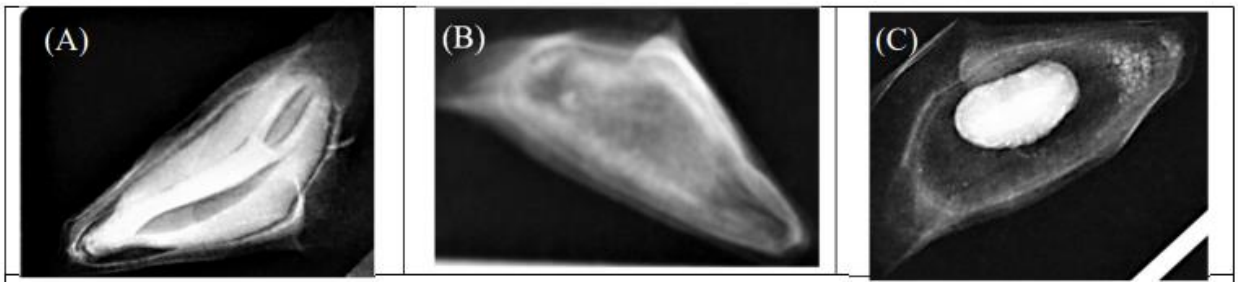


Figure 2. X-ray images of full seed (A); empty seed (B); and seed with larvea inside (C)

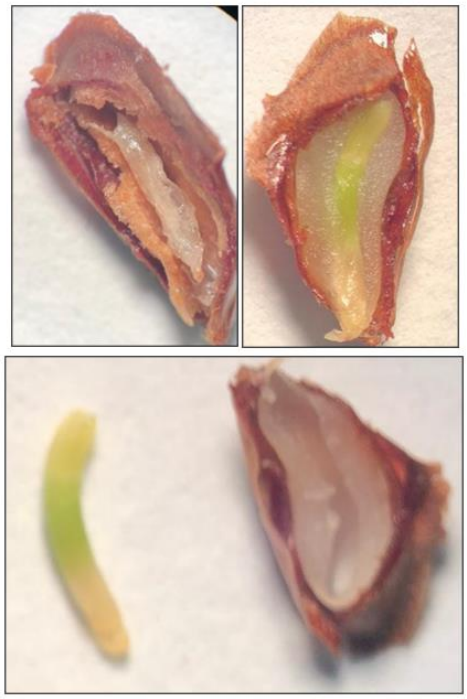


Figure 3. *A. nebrodensis* mature seeds. a. Empty seed. b. Full seed with mature zygotic embryo. c. Zygotic embryo extracted from the seed.

Furthermore, the maintenance of full-seed viability after the X-ray was evaluated through embryo germination into a hormone free SH basal salt medium.

3.3. Sterilization

- Seeds were washed with detergent for about 30 min, then flushed under running tap water for 4 hours.
- At first, seed scales were removed and treated with 70 % ethanol on a clean bench for 1 min for immature seeds and 5 min for mature seeds, then rinsed five times with sterile distilled water.
- Seeds were then immersed in 20 % (v/v) sodium hypochlorite solution to which was added a few drops of Tween 20 for 20 min and rinsed 3 times with sterile water.
- Under laminar flow hood, the seed coat was removed from the immature seeds and the megagametophytes were used as explants.
- After sterilization, mature seeds were imbibed in sterilized distilled water for 48 h under antiseptic conditions then, zygotic embryos were carefully excised from seeds using sterile forceps.

3.4. Embryos extraction

Zygotic embryos were carefully extracted from seeds within a laminar flow environment following those steps (Figure 4):

1. Carefully remove the seed coat from the megagametophytes.
2. Gently cut the endosperm to facilitate the extraction of the complete zygotic embryo.
3. Place the fully formed embryos on the selected medium in petri dishes.
4. Ensure that all steps are carried out under laminar flow conditions to maintain sterility.

For immature seeds, use megagametophytes as the explant for subsequent procedures.

3.5. Callus induction and proliferation from mature embryos

Mature embryos were excised and cultured horizontally on different culture media for callus induction. The current experiment was performed through three stages since only a limited number of seeds was available.

At first, mature seeds, previously conserved at 4 °C for 6 months, were cultured on both MS and SH media supplemented with various concentrations of BAP. Then, the suitable medium was selected depending on the callus induction rates (Figure 5).

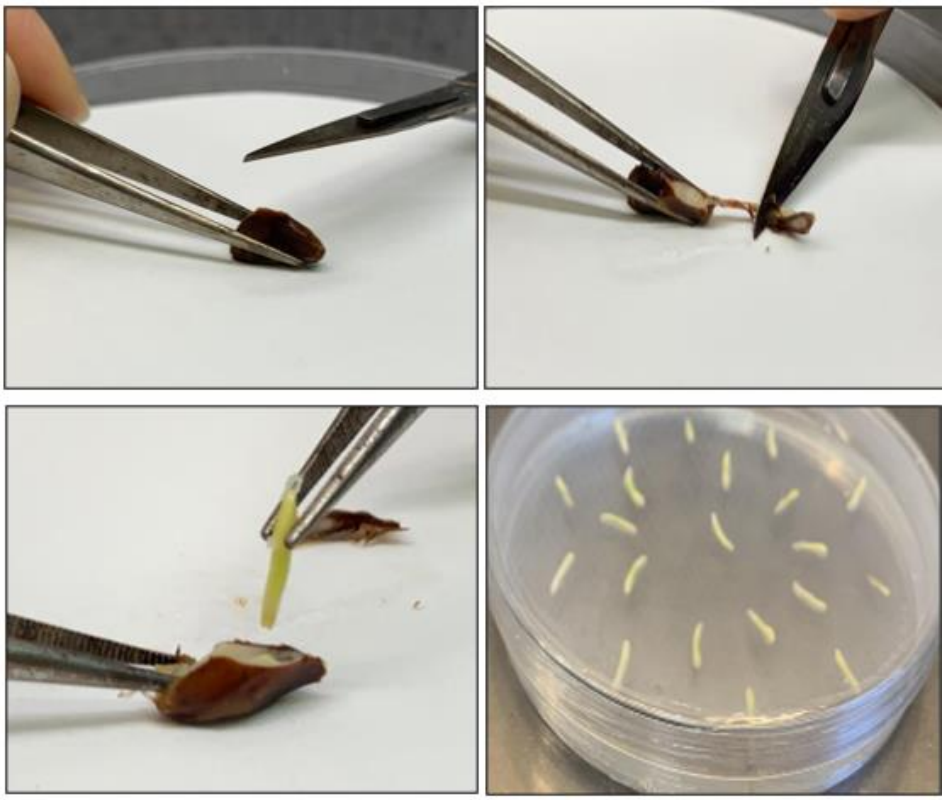


Figure 4. Embryo extraction steps.

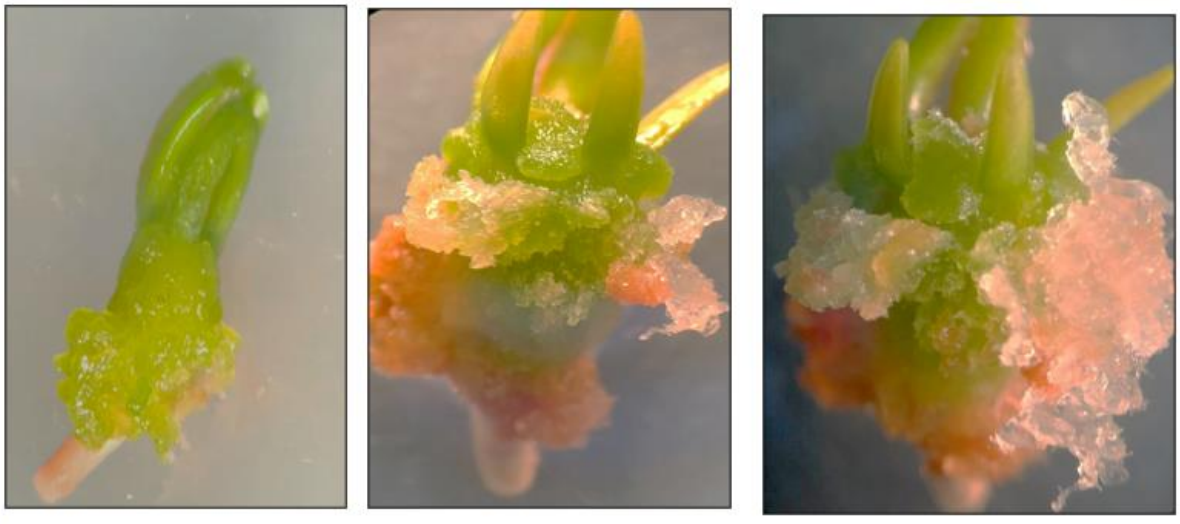


Figure 5. Callus induction from mature zygotic embryo

Once the medium was chosen, a second experiment was established in which zygotic embryos were excised from seeds of the following donor trees, with the identification number (IN), IN8, IN10, IN13 and IN21 conserved at 4 °C, for more than one year and cultured with 4,43 µM of BAP and/or 4,52 µM of 2,4-D. The effect of the donor tree was not studied in this experiment.

Later, in a third experiment, the donor tree effect on somatic embryogenesis induction was evaluated, using a fixed number of 60 mature zygotic embryos from the same donor trees of the second experiment cultured on SH media supplemented with 4,43 µM of BAP and kept in the dark at 24 ± 1 °C. Two weeks after the initiation, embryogenic callus was isolated carefully from the embryo and transferred onto a fresh medium. Embryogenic tissue (ET) was proliferated and sub-cultured onto a fresh medium every 4 weeks.

3.6. Somatic embryo development, maturation and germination

Cell lines (ET derived from each individual mature embryo) were transferred onto SH basal salt medium, supplemented with 4,27 µM ABA, PEG-4000, 8 % and 4 % of maltose. Cultures were transferred to a fresh medium every 4 weeks and kept at 24 ± 1 °C in darkness. ET formation was continuously observed under the microscope, along with the development of somatic embryos (Figure 6). Only somatic embryos with a full cotyledonary shape were used. They were isolated from callus and placed carefully on the top surface of a dry and sterile filter paper for three hours for a partial desiccation. Subsequently, two treatments were evaluated, half strength SH basal medium (G1) and full-strength MS medium (G2), both equally enriched with 1 % of both sucrose and AC, using or not a disc of filter paper placed on the top of the medium.

- Ten embryos per germination treatment were placed horizontally in Petri dishes containing 20 mL of basal medium.
- Plates were incubated at 4 °C for 21 days in the dark and then maintained at 24 ± 1 °C under light conditions (16/8 h light/dark photoperiod).

4. Encapsulation and cryopreservation assay for the embryogenic masses

Cryopreservation is a useful method for long-term preservation of plant germplasm. Small pieces (0,5 g) of embryogenic callus from IN8 and IN10 were encapsulated in alginate beads according to Micheli and Standardi protocol (2016). Later, they were washed with sterilized distilled water then submerged in 3 % sodium alginate solution (w/v). Drops of encapsulating

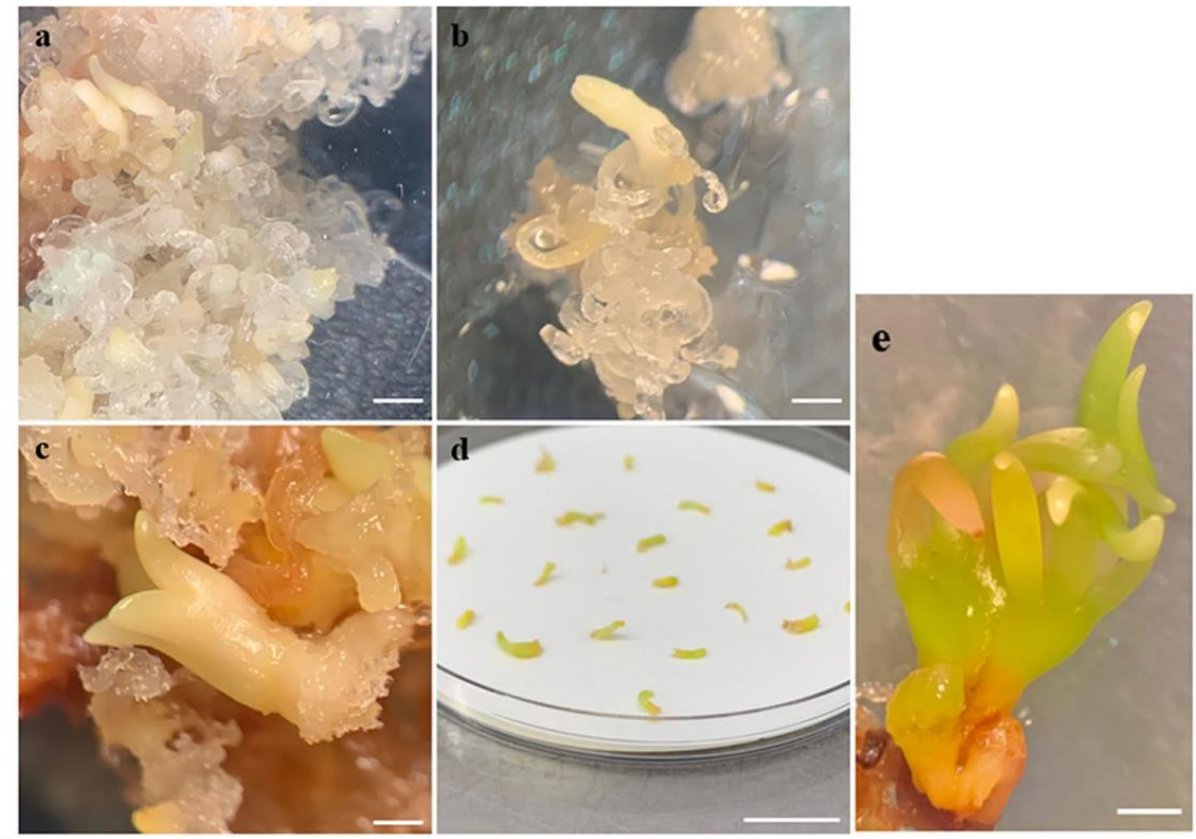


Figure 6. Proliferation and maturation of the ET. a Proliferation of the embryogenic tissue after subcultures on the proliferation medium. b Development of Pro-embryogenic masses on maturation media. c Production of cotyledons. d SE on the cotyledonary stage with a full shape. d Somatic embryo detached from the callus. e Germination of somatic embryo.

matrix containing ET were then transferred for 20 min into the MS basal medium supplemented with 11,1 mg L^{-1}calcium chloride ($CaCl_2 \cdot 2H_2O$) to obtain ET beads (Figure 7). After washing in sterile water, for cryopreservation protocol, the beads were treated with Loading solution transferred to liquid solution containing MS medium supplemented with 34,2 % of sucrose. Subsequently, a part of ET beads was treated with Plant Vitrification Solution 2 (PVS2) at 0 °C, testing different times of contact with the solution (60, 90 and 120 min) and another part of ET beads were used as control. All the ET beads were immersed in liquid nitrogen. After thawing in a water bath (2 min at 40 °C), the beads with ET were transferred onto SH medium supplemented with 4,43 µM of BAP (same medium used for proliferation) to test the viability through the ET proliferation (Figure 8).

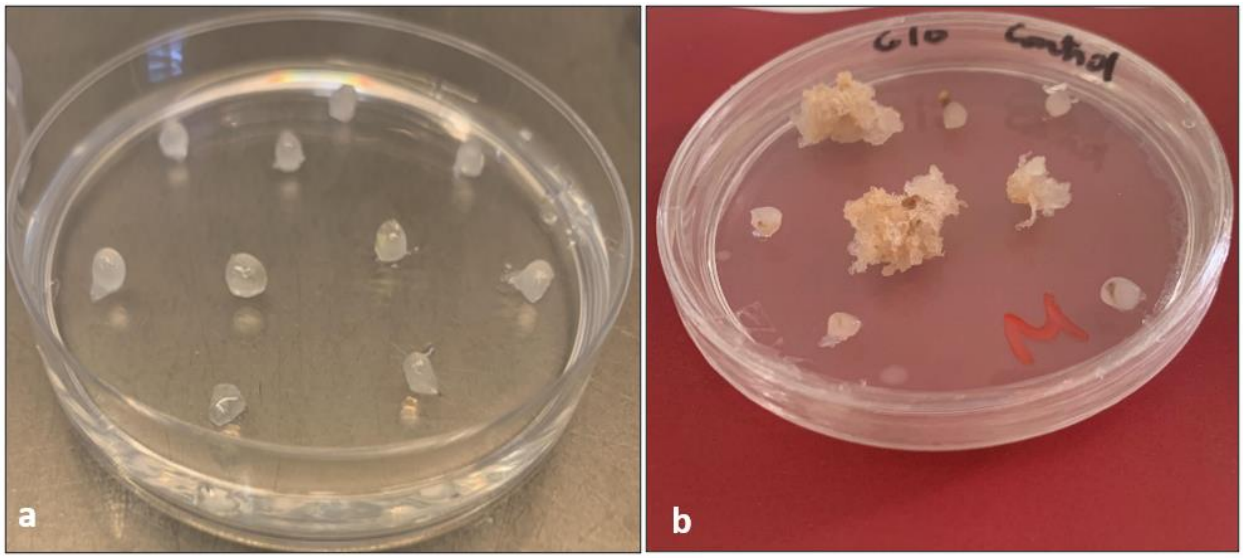


Figure 7. Encapsulation of ET. A, synthetic seed contained ET ; b, proliferation of the ET from the artificial seeds after 6 weeks in culture.

5. Conclusions

The induction and proliferation of embryogenic callus together with the maturation and germination of somatic embryos are key steps for large-scale propagation and long-term preservation of coniferous germplasm. In the project LIFE4FIR, an exclusive successful protocol for somatic embryogenis of the crtitically endangered *Abies nebrodensis*, from mature zygotic embryos is described for the first time. Employing *in vitro* cultures is difficult and laborious, but it showed the possibility to initiate somatic embryogenis as a new approach to propagate and conserve such a relict species. Investigations on SE induction from immature zygotic embryos were carried out but no relevant results were recorded. The reported findings of experiments suggest that the appropriate selection of donor trees, PGRs and basal salt media could help the maturation of somatic embryos into bipolar structures leading to emblings regeneration and acclimatization stage in the future.

Cryopreservation of embryogenic tissue could be an alternative method for long-term maintenance. Artificial seeds of ET could offer a great advantage to improve the creation of the protocol for cryo-preservation. A promising and encouraging result was obtained for encapsulated ET via sythetic seeds technology, where they showed the capacity to proliferate after encapulation.

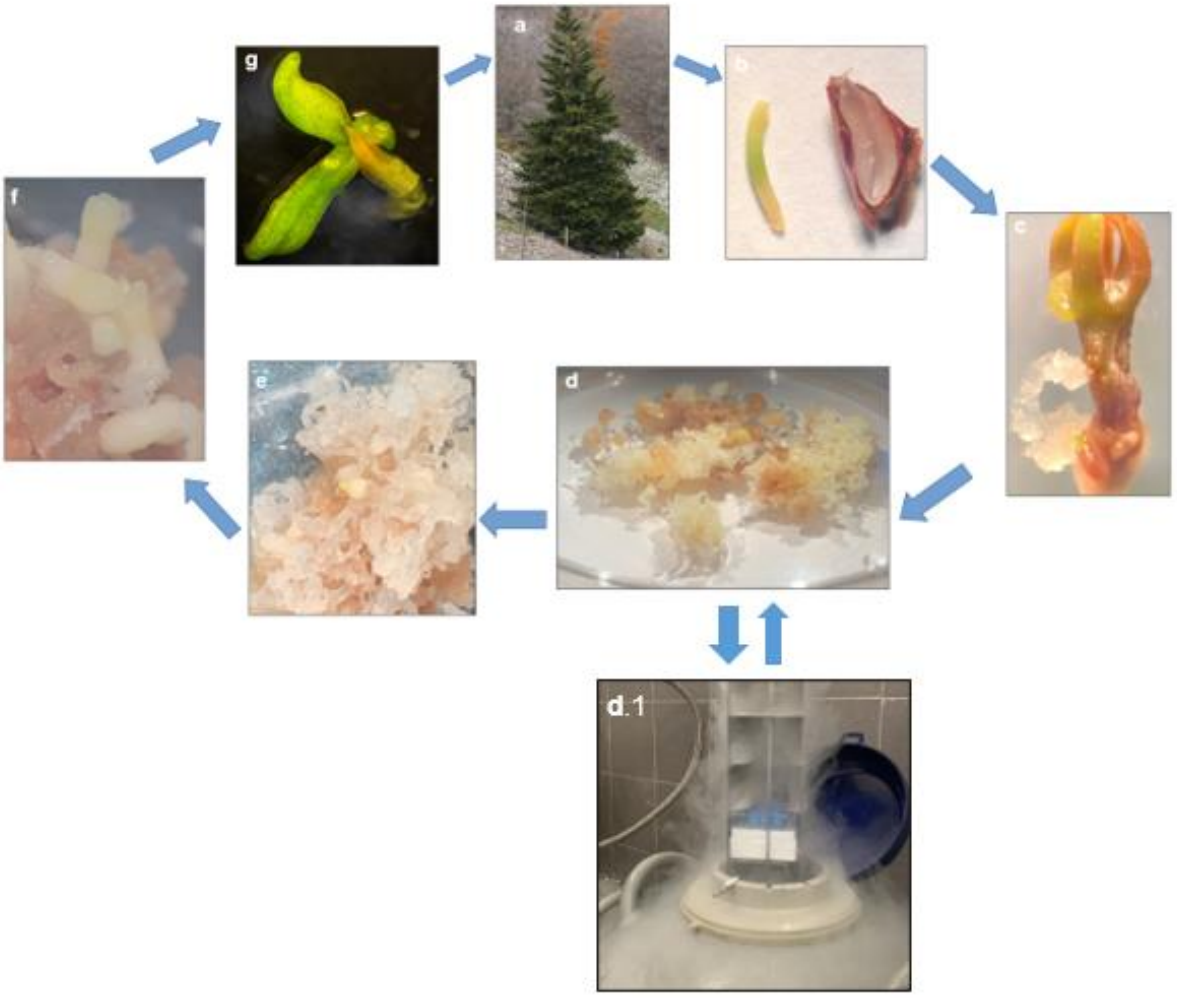


Figure 8. Schematic representation of somatic embryogenesis for Abies nebrodensis. a Sicilian fir located in the Madonie Park. b Zygotic mature embryo c Initiation of ET d Proliferation of the ET. e and f Possibilities for cryopreservation of the germplasm. g Maturation of the ET and apparition of embryogenic structure. h Formation of full SE. i Embling germination

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