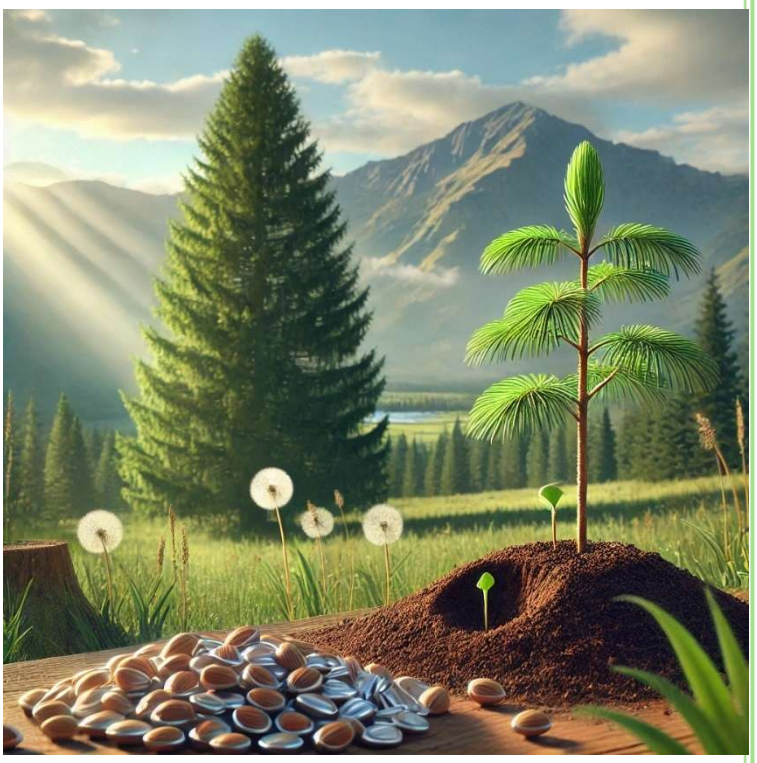
2024

The Constitution and Management of a Seed Bank and a Cryobank for the Long-Term Conservation of Sicilian Fir (Abies nebrodensis) Genetic Resources



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E-Manual

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E-Manual for the Constitution and Management of a Seed Bank and a Cryobank for the Long-Term Conservation of Sicilian Fir (*Abies nebrodensis*) Genetic Resources

1. INTRODUCTION

Abies nebrodensis, commonly known as the Sicilian fir, is a critically endangered conifer species endemic to the Madonie Regional Park in Sicily, Italy. The relic population is limited to 30 adult trees, hence the species faces significant threats from genetic erosion, habitat fragmentation, and poor natural regeneration. Abies nebrodensis, like other conifers, produces many seeds without embryos. For effective conservation, it is essential to select only full seeds containing complete embryos. Conservation efforts for A. nebrodensis are crucial to prevent its extinction and preserve its genetic diversity. This e-manual includes and describes the two method applied for ex situ conservation of this species, a Seed bank and a Cryobank, that have been established in the Museum of *Abies nebrodensis* (MAN) in Polizzi Generosa (Palermo), Italy. This strategy developed for *A. nebrodensis* can pave the way to similar initiatives for other critically endangered conifer species.

2. SEED-BANKING

Seed banks are a vital part of ex situ plant conservation strategies of forest species, involving the storage of seeds at low temperatures (-18°C). Seed samples are small, easy to handle, can maintain viability for long periods and are relatively cheap for the long-term storage. In the world, many Countries have established Seed banks for crops and forest trees, highlighting the importance of this conservation method.
Seed banking provides several advantages, such as:

- **Easy Storage**: Requires limited space, minimal manual labor, and low cost to maintain large collections.
- **Preservation**: Protects plants from habitat destruction, diseases, and predators.
- **Restoration**: Facilitates the restoration of species in suitable habitats.

2.1 Material

2.1.1 *Plant Material*

Mature cones collected in October from *A. nebrodensis* trees.

2.1.2 *Laboratory Equipment*

- **Mesh, Fine Textile** - Used for the initial cleaning of collected cones and seeds, removing larger debris and impurities. Fine mesh material that allows the initial cleaning by separation of seeds from larger particles without damaging the seeds.

- **Sieve, Fine Mesh Strainer** - Helps in further separating seeds from smaller debris and dust particles. Fine mesh with varying pore sizes, depending on the seed size.
- **Moisture Analyzer Instrument -** High-precision analyzer with capabilities to measure the seeds moisture content quickly and accurately.
- **Weighted Balance** - High-precision balance with a readability of up to 0.001 grams, for precise weighing of seeds to record their weight accurately
- **Petri Dishes, Forceps and Others** - Petri dishes of different sizes. Stainless steel forceps for handling seeds. Filter paper.

2.1.3 *Seeds X-ray Analysis*

- **Plastic Square Well Plates** - Plates with multiple wells (20x20 cm) to hold individual seeds in place during the X-ray process. Used to organize seeds during X-ray analysis.
- **X-ray Film** - High-resolution X-ray film suitable for detailed imaging.
- **X-ray Apparatus Instrument** - Advanced X-ray apparatus capable of producing high-resolution images with adjustable settings for various seed types, to detect the presence of embryos inside seeds.
- **Laboratory Plastic Tray** - For handling and organizing seeds during X-ray and other laboratory procedures. Trays of durable plastic material (45 X 35 X 7.5 cm), suitable for repeated use in laboratory environments.
- **Development Solution** - Standard development solution suitable for use with X-ray films; used for developing the X-ray films after exposure.
- **Fixation Solution** - Standard fixation solution used in X-ray film processing to develop X-ray images to prevent further changes and ensure stability.
- **Orbital Shaker** - Ensures even exposure of the film to solutions. Adjustable speed and timing settings to accommodate various laboratory procedures.
- **Film Viewer Screen** - High-resolution screen with backlighting to enhance visibility of X-ray images to identify viable seeds with embryos

2.1.4 *Seeds Viability Validation*

- **Tetrazolium Chloride Solution (TTC)** - 2,3,5-triphenyl tetrazolium chloride powder to prepare a solution at 1%, used to assess seed viability by staining viable tissues.
- **Oven -** to complete TTC reaction
- **Stereoscope -** for the visual observation of embryos

2.1.5 *Seed Conservation*

- **Jar Labeled with Seed Sample Information -** For storing seeds with detailed information to ensure accurate record-keeping.
- **Freezer Chamber** - Serves as Seed bank with controlled storage temperature conditions. Capable of maintaining precise temperature (-18°C) and humidity settings for optimal seed preservation.

2.2 Methods

2.2.1 Cleaning of Seeds

Cleaning: Extract seeds from cones and clean them using fine mesh and textile to remove impurities.

2.2.2 Screening of seeds

*X-ray technique to evaluate the full seeds**

- Place seeds in plastic square well plates (20x20 cm) and expose them to X-rays using the X-ray apparatus. For *A. nebrodensis*, the best resolution of the image was achieved by applying 25 kV, 3 mA (soft X-rays) at a distance of 45 cm from the X-ray source for 2 min.
- Wash X-Ray film in the development solution for 4 min and then in fixation solution for 3 min on the shaker at 20 rpm. Then, wash the film under tap water for 3-5 sec and examine it on the film viewer screen. Check the X-ray image of each seed for the presence of the embryo (Fig.1) and select only full seeds with healthy and undamaged embryos.

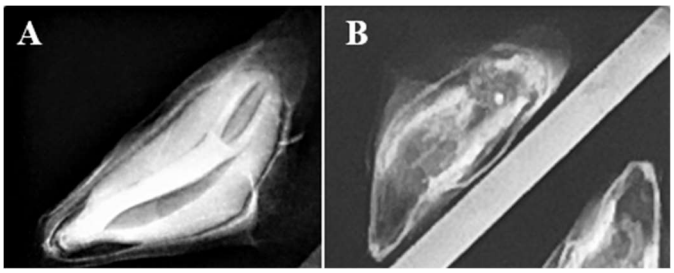


Fig 1. X-ray images of full seed (A) and empty seed (B).

2.2.3 Visual Observation

Open and check under the stereoscope a sample of seeds after X-ray to confirm the presence of embryo and have a validation of the technique (Fig 2.).

2.2.4 Seed viability by TTC test

- Soak a sample of seeds in water for 24 hours. Cut the seed coat (Fig. 3a) at two sides (Fig. 4 A) to allow the penetration of TTC solution.
- Immerse seeds in 0.1% (w/v) TTC in 50 mM Tris-HCl buffer (pH 7.6) for 24 h in darkness at 30 °C. After this treatment, wash the seeds twice with distilled water and place in petri dishes with moist filter paper.

  Cut longitudinally, remove seed coat, and extract the embryo to observe the development of red color. The red color of zygotic embryo is the main indicator that the seed was alive (Fig. 3C).

Note: *The X-ray technique is essential for selecting viable seeds by detecting the presence of embryos. This method allows the removal of empty seeds, ensuring that only seeds containing well-formed embryos are stored in the Seed bank

Fig.3. Seeds prepared for the staining process: (A) cross section in the seeds before TTC test;(B) Tetrazolium staining patterns (white non-viable seed; red viable seed); (C) Non-viable embryo (1) viable embryo (2, 3, 4).

2.2.5 Seed Preparation for Conservation

- Measure moisture content of full seed sample using 0.1g (~3 seeds) with moisture analyzer apparatus (Fig. 4). For *A. nebrodensis* the average of moisture content is from 6.3% to 10%.**

Fig. 4. Determination of moisture content by Moisture Analyzer (Mettler-Toledo AG, Laboratory & Weighing Technologies, Greifensee, Switzerland)

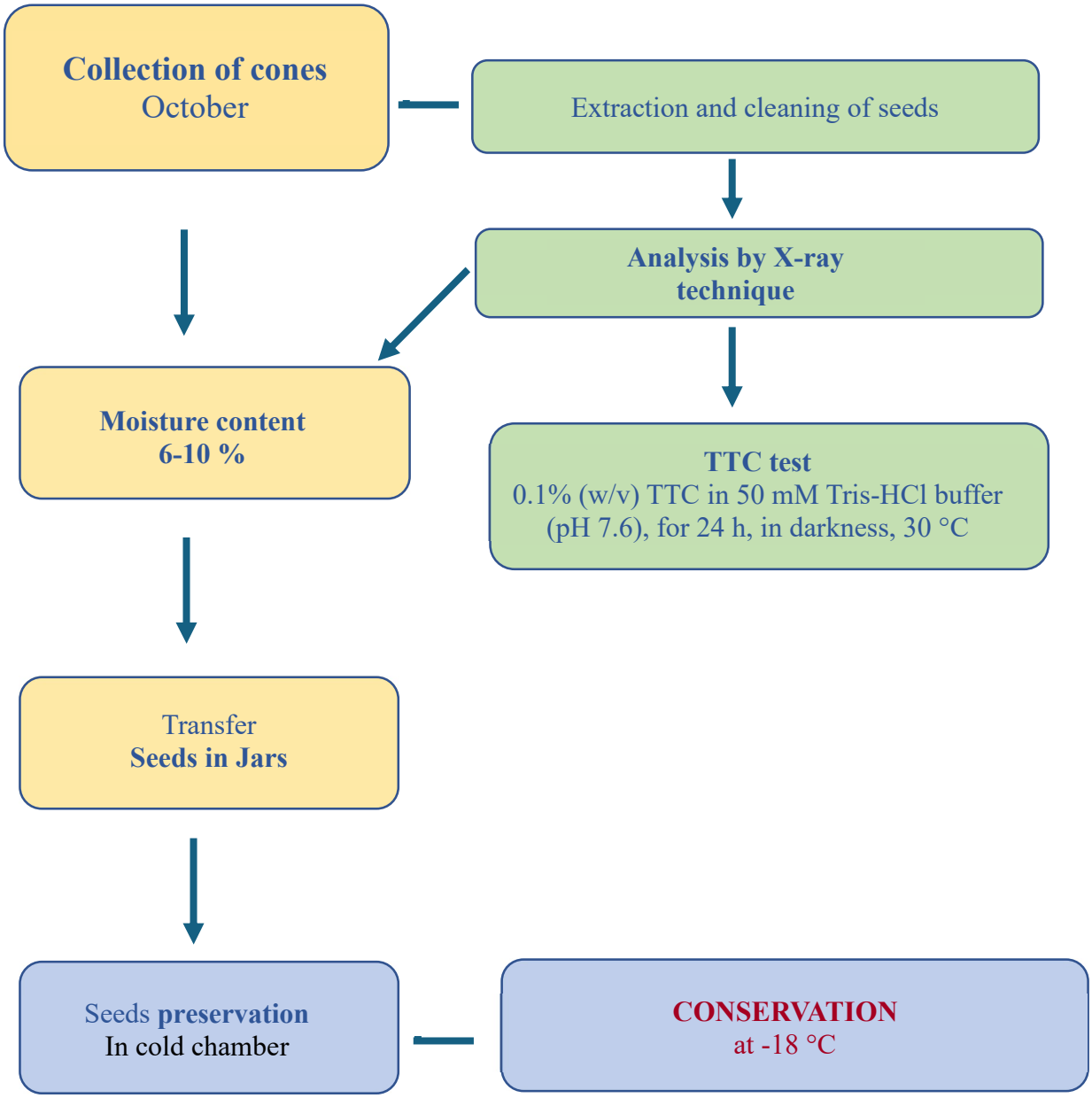
- Weigh and count the full seeds to ensure the storage of high quality seeds.
- Place the seeds in jars labelled with information on***: plant number, collection year, weight (gr), seeds number, starting date of conservation, and location of seed bank (Fig. 5).
- Transfer the labelled jars to a freeze chambers (-18°C) for storage (Fig.6)

Notes:
** The optimal moisture content of seeds ensures the viability during the storage.
*** Labelling: Accurate labelling and record-keeping are crucial for the management of samples in Seed bank.

Fig. 5. Jar labeled with seed sample information

Fig. 6. Seed bank of A. nebrodensis with some seed samples

2.2.6 Final Protocol for Seed Conservation at Low Temperature (-18°C)



3. CRYOBANK FOR LONG-TERM CONSERVATION

A cryobank is a facility used for the long-term preservation of vegetal material at ultra-low temperatures, such as the-196°C of liquid nitrogen. Cryopreservation is the storage of cells, tissues and organs from in vitro culture (shoot tips, embryogenic callus, somatic embryos), as well as from in vivo collected material (pollen, seeds, embryonic axes and dormant buds) in liquid nitrogen. Under these conditions, plant materials can be conserved for unlimited time in absolute sanitary and genetic safety due to suspension of all the biochemical and physical cell processes. In addition, long-term storage in liquid nitrogen eliminates the problems (large spaces, costs of management) of maintaining to store collections in field or in vitro.
Cryobanks are crucial for conserving the genetic diversity of endangered species like *Abies nebrodensis*; in the Cryobank of this species are preserved three types of explants, i.e., pollen, zygotic embryos and embryogenic callus lines.

3.1 Material

3.1.1 Plant Material

Male cones of pollen collected in May and mature cones collected in October from *A. nebrodensis* trees.

3.1.2 Laboratory Equipment and Chemicals

- **Fine Mesh Strainers -** to remove the pollen grains from anthers
- **Moisture Analyzer instrument -** High-precision analyzer with capabilities to measure the pollen and zygotic embryos moisture content quickly and accurately.
- **Weighted Balance** - For precise weighing of collected materials. High-precision balance with a readability of up to 0.001 grams.
- **Tetrazolium Chloride Solution (TTC)** - 2,3,5-triphenyl tetrazolium chloride powder to prepare a solution to assess pollen and zygotic embryos viability.
- **Paint brush** (size 08; 5 mm) – to dust pollen used in assays
- **Microscope slides and coverslips –** used in the TTC test
- **Plastic pipette** (1 mL) – use to drop the TTC solution
- **Oven** - to complete TTC reaction
- **Microscope and Stereoscope** - for the visual observation of pollen
- **Petri dishes** (Ø 60 mm and 90 mm)
- **Solid medium for germination: *pollen* -** boric acid, sucrose, and plant agar; ***zygotic embryos*** - Murashige and Skoog medium, 1962 (MS); **embryogenic callus -** Schenk and Hildebrandt, 1972 (SH) medium, 6-benzyladenine (BA), casein, and glutamine.
- **Beaker** of different volumes – to use for the seeds sterilization
- **Sterilized distilled water** -for soaking and washing of the seeds
- **Sodium hypochloride** – for sterilization of the seeds
- **Tween® 20** - use as a detergent and emulsifier
- **Ethanol 70%** - for sterilization of the seeds
- **Forceps, Scalpels, and Filter paper.**
- **pH meter –** to measure the pH of different culture medium.
- **Autoclave -** used to sterilize material and equipment for tissue culture

- **Laminar flow cabinet** - Ensures a sterile environment for handling samples to prevent contamination. Equipped with HEPA filters to provide a clean air environment.
- **Growth chamber** - temperature 25 ± 2°C.
- **Water bath** – to thaw the cryopreserved explants.
- **Cryopreservation Solutions (Plant Vitrification Solution 2- PVS2;** Sakai et al, 1990**) –** Used to prepare samples for cryopreservation process. Includes following components: 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide in MS medium liquid, and 0.4 M sucrose.
- **Washing solution –** Used to remove the PVS2 traces - Includes following components liquid MS medium and sucrose.
- **Cryovials** - Sterile, high-quality vials capable of withstanding ultra-low temperatures for storing biological material at cryogenic temperatures. Capacity 2.0 mL
- **Cryoboxes and racks -** Durable, autoclavable plastic boxes for long-term storage. Resistant stainless-steel racks to support cryoboxes inside the dewar.
- **Liquid Nitrogen Storage Tanks –** Thermal dewars designed to maintain liquid nitrogen at stable, ultra-low temperatures. Tanks are typically made of stainless steel and have a capacity ranging from 20 to 500 liters. Used for long-term storage of cryovials.

3.2 Methods

3.2.1 Collection and Preparation for Pollen Cryopreservation

- After collection remove the pollen from anthers, sieve with fine mesh strainer (Fig. 7A and 7B).
- Determine moisture content by Moisture Analyzer instrument weighing at least 0.1 g of pollen. The moisture content fresh pollen of *A. nebrodensis* ranges from 8 to 10% (Fig. 7C)

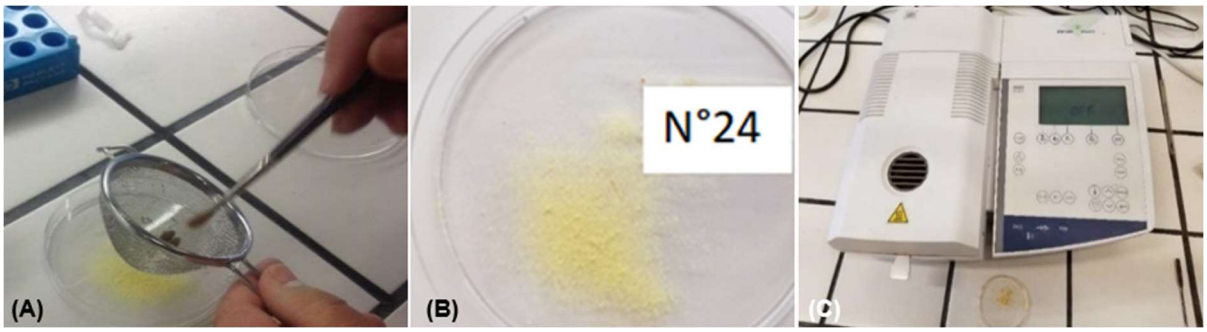


Fig.7. Pollen sieving (A); Fresh pollen (B); Evaluation of the moisture content (C)

- Transfer samples of pollen in the cryovials, place them in the cryoboxes.
- Place cryoboxes inside the rack and immerse them directly into liquid nitrogen.
- After storage in liquid nitrogen, thaw the cryovials containing the pollen under a laminar flow cabinet for 2 hours at room temperature.
- Evaluate viability and germinability using TTC and in vitro germination tests before and after cryopreservation.

Pollen viability by TTC test

- Prepare 1% TTC by adding 200 mg of 2, 3, 5 triphenyl tetrazolium chloride and 12 g of sucrose in 20 mL distilled water.
- Place two drops of this mixture on a microscope slide, use the paint brush to dust the pollen, and cover it with a coverslip.
- Incubate the pollen samples in the oven (30°C) under darkness for 24-48 h.
- Check the viability under the microscope; pollen grains stained red colour are scored as viable (Fig. 8 a-c).

In vitro germination of pollen

- Prepare the in vitro germination medium as follows: boric acid (50 mg/L), sucrose (15 g/L) and plant agar (6 g/L). Sterilize the medium in autoclave at 120 °C for 20 min, pour 10 mL of medium inside the petri dishes (Ø 60 mm) under the laminar flow cabinet. Leave them until the agarized medium is solidified.
- Use the paint brush to dust the pollen over the solid medium. Place the petri dishes in darkness in the growth chamber at 25 ± 2°C for 24-48 h.
- Check the germinability of pollen under the stereoscope. Observe the elongation of the pollen tube; pollen tubes that elongate to a length at least two times the diameter of the pollen grain, are considered germinated (Fig 8 d-e).

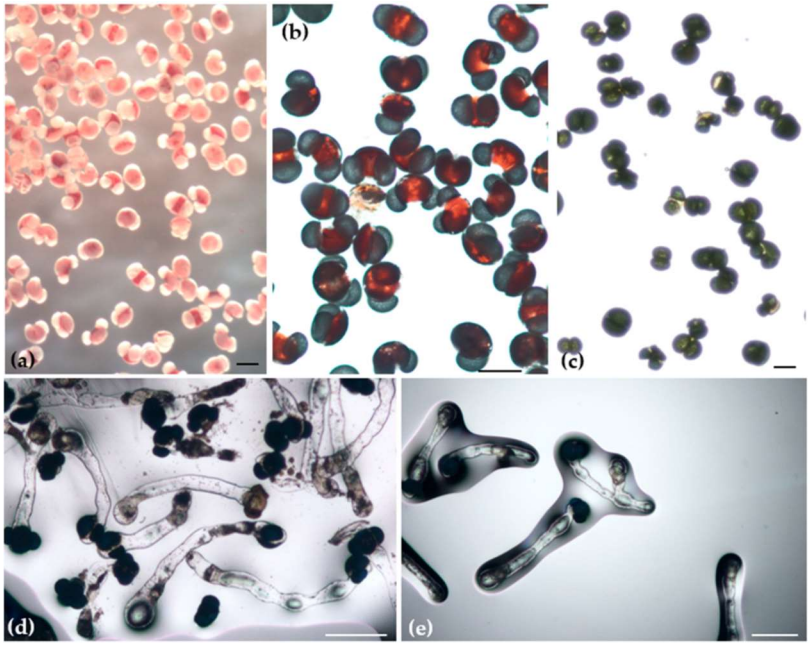
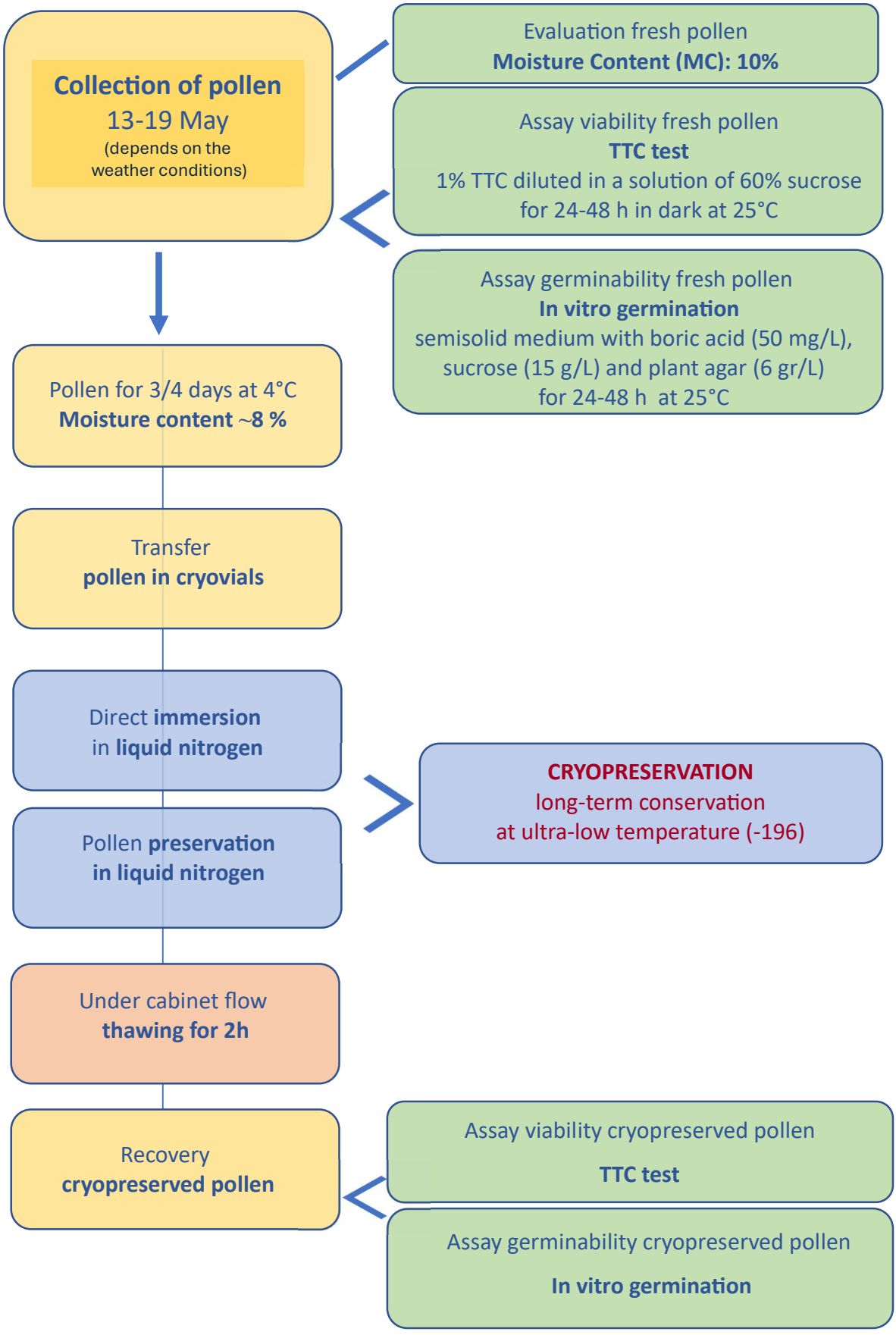


Fig.8. Cryopreserved pollen grains of Abies nebrodensis: viable pollen grains after TTC under stereomicroscope (a) and under microscope (b); non-viable pollen grains under optical microscope (c); in vitro germination of pollen grain under optical microscope e (d,e) (Bars, 100 µm).

3.2.2 Final Protocol for *A. nebrodensis* Pollen Cryopreservation



3.2.3 Collection and Preparation of Zygotic Embryos for Cryopreservation

- Clean the collected materials to remove seeds impurities (see 2.2)
- Select full seeds by X-rays analysis (see 2.2)
- Seeds sterilization - For the sterilization, under the cabinet laminar flow, treat full mature seeds with 70% ethyl alcohol for 5 min, and rinse with sterile distilled water 3 times. Then, treat seeds with 20% v/v sodium hypochlorite with 3 mL of Tween 20% solution for 20 min, and rinse with sterilized water 3 times.
- After sterilization, maintain the seeds in sterilized water for 48h under aseptic conditions.
- Excise the zygotic embryos from the seeds, under the cabinet laminar flow (Fig 9)

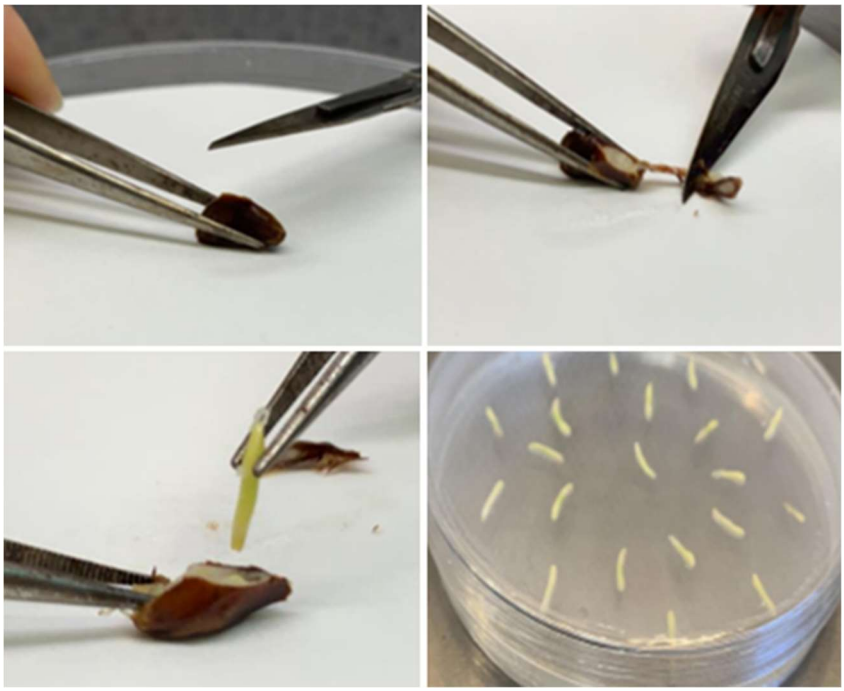


Fig. 9. *Excision* steps of zygotic embryos

- Transfer zygotic embryos in the cryovials, add 1.2 mL of PVS2 and put them in the cryoboxes (Fig. 10A).
- Insert cryoboxes in the rack and then immerse the rack directly into liquid nitrogen (Fig. 10B)

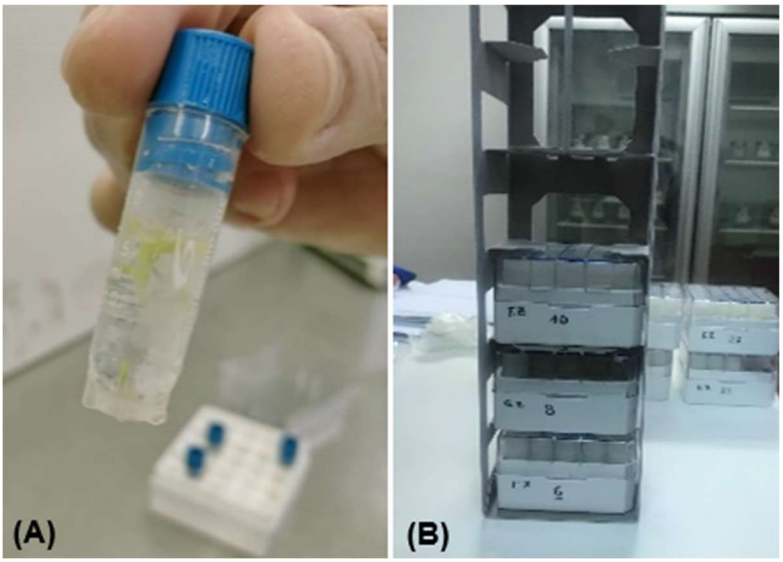


Fig. 10. Cryovial containing embryos in PVS2 (A); cryoboxes in rack (B)

- After storage in liquid nitrogen, thaw the cryovials containing the zygotic embryos in a water bath at 40 °C for 1 min.
- Treat the cryopreserved zygotic embryos for 20 min with washing solution. (Washing solution - liquid MS medium containing 1.2 M sucrose)
- Evaluate viability using TTC and vitro germination before and after cryopreservation.

Zygotic embryos viability test by TTC

- Prepare 0.1% TTC in 50 mM Tris-HCl buffer (pH 7.6),
- Pour the TTC inside the vials (2 mL) containing five zygotic embryos
- Incubate the vials in the oven at 30°C under darkness for 24 h.
- Check the viability; red colour of zygotic embryos is the indicator of viability (Fig.11 C)

Zygotic embryo In vitro germination

- Prepare the in vitro germination medium as follows: hormone-free Murashige and Skoog, containing sucrose 20 g/L and agar 7 g/L; pH 5.8.
- Pour 10 mL of MS medium in petri dishes (Ø 60 mm)
- Place five zygotic embryos for petri dishes and maintain at 24 °C under a 16-h photoperiod in growth chamber.
- Evaluate the germination after 3 weeks of culture (Fig.11 D-E)

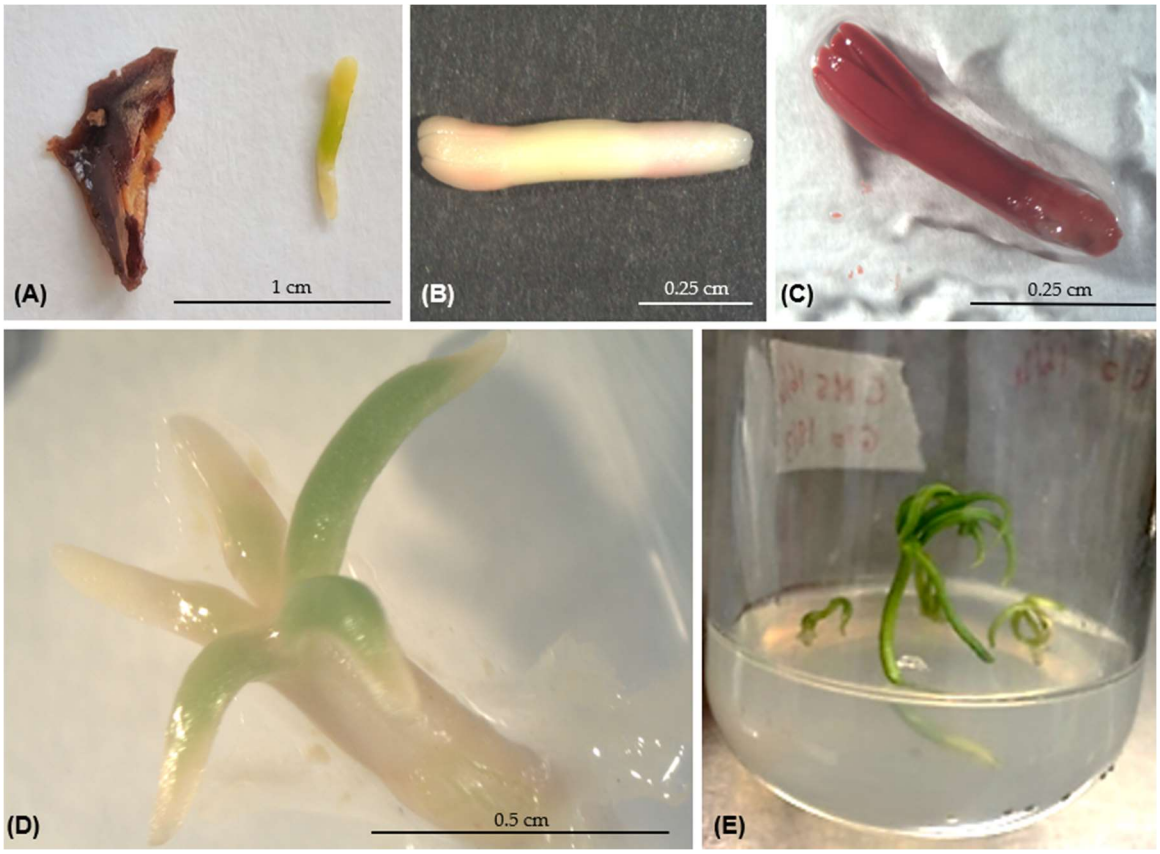
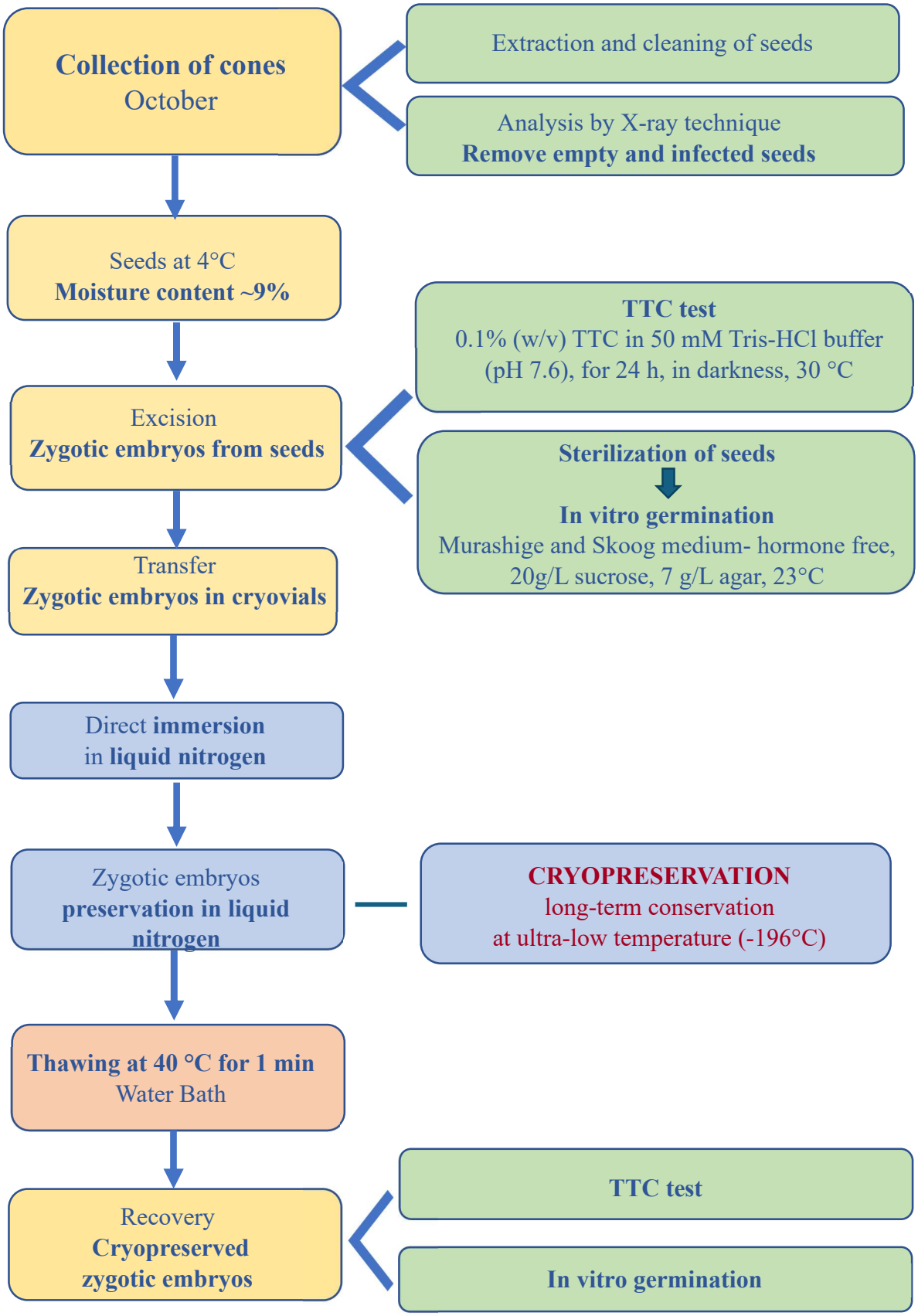


Fig. 11. Untreated zygotic embryo: (A), cryopreserved zygotic embryos after TTC test resulting non-viable (B) and viable (C); germination of zygotic embryos 7 (D) and 20 days (E) after the recovery from cryopreservation.

3.2.4 Final Protocol for *A. nebrodensis* zygotic embryos conservation in Cryobank (-196°C)



3.2.5 Collection and Preparation for Embryogenic Callus Cryopreservation

- Clean the collected materials to remove seeds impurities (see 2.2)
- Select full seed by X-rays analysis (see 2.2)
- Sterilized seeds (see 3.2.2)
- Excise mature zygotic embryos from the full seeds under cabinet laminar flow
- Prepare the induction medium as follows: Schenk and Hildebrandt (SH) medium, 1mg/L BA, casein 1 g/L, glutamine 500 mg/L, sucrose 20 g/L and 7 g/L agar; pH 5.8.
- Pour 25 mL of the induction medium in petri dishes (Ø 90 mm)
- Place about 25 zygotic embryos in petri dishes for embryogenic callus induction
- Maintain the embryogenic callus at 24°C in dark conditions.
- Subculture embryogenic callus on fresh medium, every four weeks, to obtain the proliferation of callus (Fig. 12).

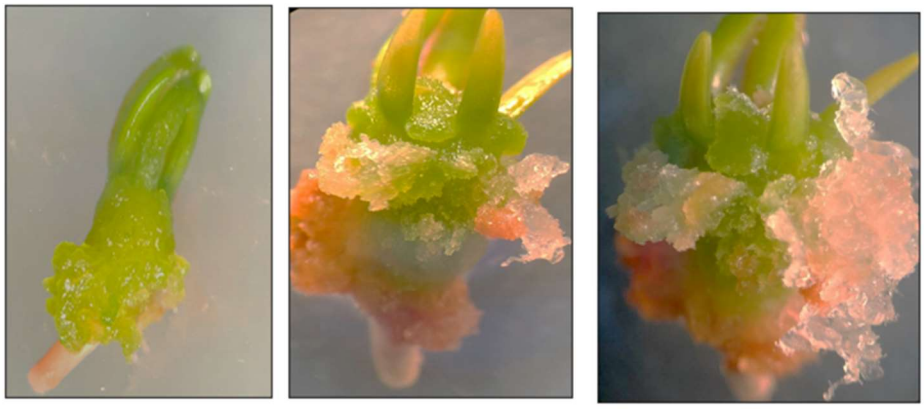


Fig. 12. Callus induction and proliferation from mature zygotic embryo

- Encapsulate portion of embryogenic callus in alginate-calcium beads as describe by Standardi and Micheli (2012) (Fig. 13A-C)
- Transfer the beads to sterilized filter paper inside Petri dishes, then place in glass jars containing sterilized silica gel for 1-5 hours for beads dehydration (Fig 13D).
- Place the dehydrated beads in cryovials and immerse in liquid nitrogen (Fig 13E-F).
- Thaw the cryopreserved beads with embryogenic callus in a water bath at 40°C for 2 min, then treat with a washing solution for 20 min.
- Place the beads on fresh SH regrowth medium supplemented with 1 mg/L BA, 1 g/L casein, 500 mg/L glutamine, 20 g/L sucrose, and 7 g/L agar; pH 5.8.
- Check the regrowth ability of encapsulated embryogenic callus when the callus broke through the gel of the bead (Fig. 13G)

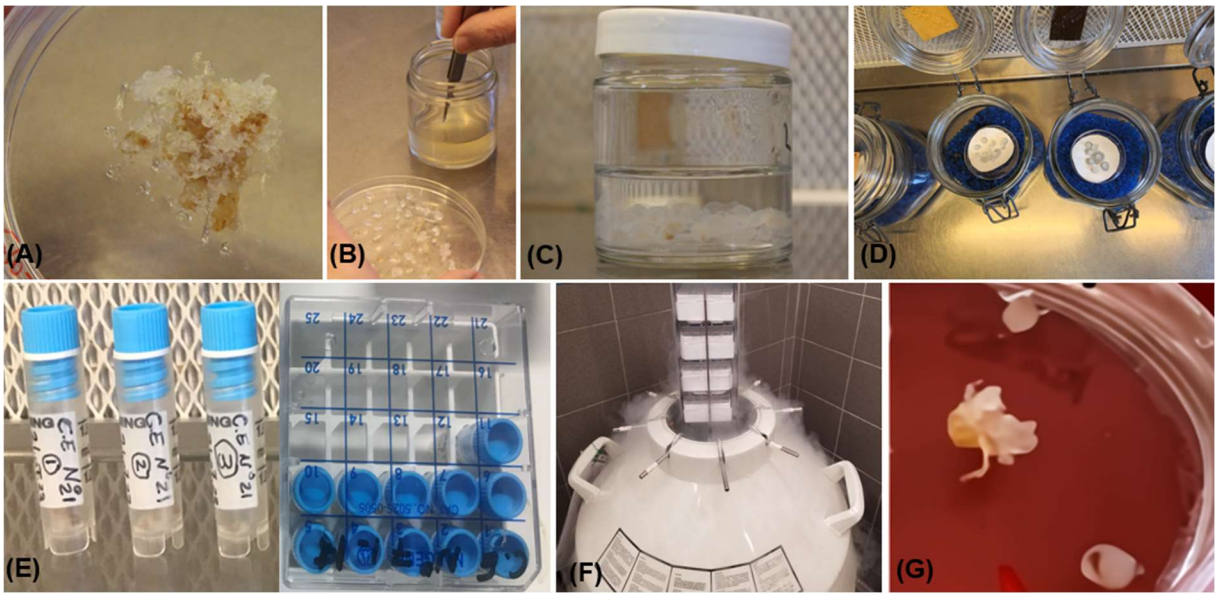


Fig. 13. Flowchart depicts the major steps for the cryopreservation of A. nebrodensis.embryogenic callus. Embryogenic callus-EC (A); preparation of EC beads (B and C); dehydration of EC beads on silica gel (D); dehydrated EC beads in cryovials; cryovials inserted in cryobox (E); immersion of the rack in liquid nitrogen (F); regrowth of cryopreserved EC (G).

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3.2.6 Final protocol for the cryopreservation of *Abies nebrodensis* embryogenic callus