**LIFE4FIR – Project LIFE18 NAT/IT/000164**

**"Decisive in situ and ex situ conservation strategies to secure the critically endangered Sicilian fir, *Abies nebrodensis*"**

# Report of a complete protocol for *A. nebrodensis* seed and excised zygotic embryo conservation at low (-18°C) and cryogenic (-196°C) temperatures, respectively.

## Action A.1


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DELL'AGRICOLTURA, DELLO SVILUPPO RURALE
E DELLA PESCA MEDITERRANEA

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## 1. Introduction

The LIFE4FIR project aims to safeguard the natural population of *Abies nebrodensis* and to increase its genetic diversity. Actually, its actions are not limited to reduce the risk of extinction by improving and developing a model of protection but also to promote the ex situ conservation by the establishment of a germplasm bank (seed bank and cryobank) in the Museum of *A. nebrodensis* at Polizzi Generosa, in Sicily.

The project proposes many specific actions to support and preserve *Abies nebrodensis* population, in particular Action 1.4, that indicates the conservation at low temperature (-18°C) and the application of cryopreservation protocols.

For preservation of the plant biodiversity there are various conservation methods but the most appropriate strategy for the complement of plant conservation germplasm is combining approaches of ex situ and in situ procedures.

In this Life project, the ex situ conservation of plant species, considered an effective process against extinction in the wild while being available for research, reintroduction and restoration is presented as an important tool for the establishment of ex situ germplasm conservation programs.

## 2. Importance of Seed Conservation (seed bank and cryobank)

The storage of seeds as plant reproductive structures is one of the most common and valuable ex situ approaches since seed samples are small in size, ease of handle, require less maintenance and frequently maintain viability for long periods.

Traditionally, seed-propagated plant species can be conserved in seed banks while those that are vegetatively propagated can be preserved in field collections or in in vitro cultures. Seed banking has considerable advantages over other methods of *ex situ* conservation due to easy storage, economy of space, relatively low labour demands and consequently, the capacity to maintain large samples at an economically viable cost. The last evaluation of FAO (2010) showed that there are more than 1,750 gene banks (also termed seed banks) holding a total of 7.4 million accessions.

Many factors increased the popularity of seed bank as a convenient means for the conservation:

- First, seed banks provide immediate access to plant samples, allowing researchers and conservation biologists to evaluate them for properties such as new sources of medicines, nutrition, and genes.
- Second, the availability of plant germplasm in seed banks facilitates scientific study that could provide helpful information for conserving the remaining natural populations of the species.
- Third, and perhaps the most important, plants conserved in seed banks are immune to habitat destruction, diseases, and predators. They can be used to reinstate species into existing, suitable habitats where they were once present, or to augment the diversity of small, genetically depleted populations.

For the optimum conservation of seeds, is required to define adequate moisture content, appropriate storage temperature, and careful production of quality seed to ensure the greatest longevity (storing them in hermetically-sealed containers).

Beside the cryopreservation (−196 °C) offers more alternatives to preserve different types of plant materials from in vivo and in vitro. 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 (LN, –196 °C). 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 LN eliminates the problem of maintaining big spaces required to store in vitro a backup of abundant collections, the potential risks of contamination during the storage, and the somaclonal variation for some species.

For non-orthodox seed species, cryopreservation is the only method available for long-term conservation. In the case of intermediate seed-propagated species, seeds are partially desiccation tolerant and, therefore, the whole seed cryopreservation is the first option to be tested. The water content of the seeds at the moment of immersion in LN must be regarded as the most critical factor since the seed survival strictly depended on avoidance of intracellular ice formation. Therefore, seed moisture content and germination conditions need to be optimized to maximize freezing tolerance and recovery after cryopreservation process.

## 3. Steps to Achieve Protocol for Conservation of Seeds and Zygotic embryos of *A. nebrodensis*

### *3.1. Cones collection, extraction, cleaning, and moisture content of seeds*

The cones from the *Abies* trees (6, 7, 8, 10, 12, 13, 19, 21, 22 and 27) were harvested in October and then dried to equilibrium in a controlled environment. Mature seeds were cleaned from any remaining impurities (Fig. 1A) and their moisture content (Fig. 1B) was measured using> 0.1g (~3 seeds). The average of moisture content was 6.3 % and then the seeds were stored at +4ºC till the beginning of the experiments.

(A) (B)

*Fig. 1. Extraction and cleaning of seeds (A); Determination of moisture content by Moisture Analyzer (B)*

### *3.2. X-ray technique to evaluate the full seeds*

*Abies nebrodensis* trees produce large quantities of empty seeds, that are without embryo development. For this reason, the application of the X-ray method can be highly effective tool to evaluate the presence or absence of embryos inside the seed. Based on X-ray image, seeds are found empty or attacked by insects or diseases should be removed and considered not valid for preservation and not included in further assays.

- X-ray image

Seeds were prepared in the laboratory in plastic square well plates (20x20 cm). All seeds were X-ray photographed and 100 seeds per treatment (Fig. 2A) were individually evaluated with X-

ray analysis. The radiography was done with a Gilardoni radio light, Lecco, Italy, and after preliminary test, the best result in term of time, dose…etc. was achieved using X-ray film exposed to 25 kV, 3 mA (soft X-rays) at a distance of 45 cm from the X-ray source for 2 min (Fig. 2B).

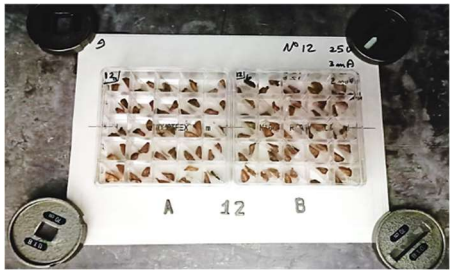
(A)

100 Seeds in square well plates

(B)

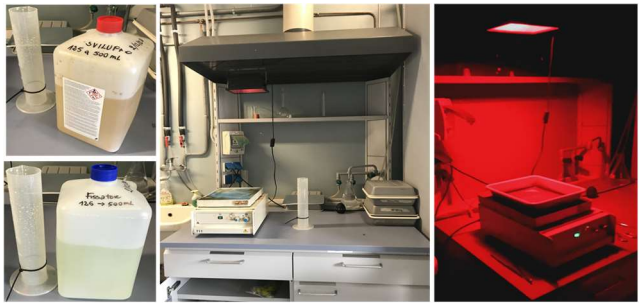

Selecting appropriate X-Ray

(C)



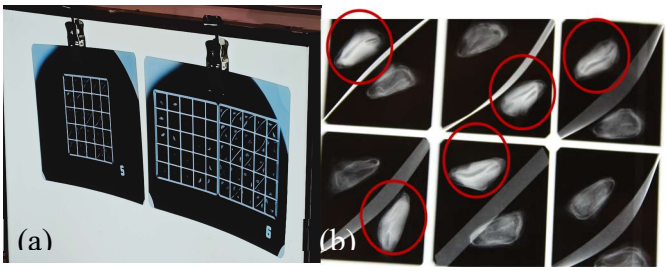

Seeds on Carestream X-Ray Film

(D)



Development of X-Ray Film

(E)




a. Checking of the seeds on X-Ray film viewer Screen
b. Empty seeds and full seeds (in the red circle)

*Fig 2. X-Ray application on A. nebrodensis seeds*

The film was developed in Carestream X-Ray Film (Fig. 2C). After X-ray application, the films were washed in the development solution for 4 minutes and then in fixation solution for 3 min

on the shaker at 20 rpm (Fig. 2D). Finally, the films were washed under tap water at 3-5 seconds and were examined by film viewer screen (Fig. 2E).

X-ray images were verified for the presence of endosperm and embryo. Embryo morphology was examined as either normal or absent in the seeds (Fig. 3). A normal seed contained a perfect embryo, embryo cavity, and endosperm. Three basic structures might be observed in *A. nebrodensis* seeds: embryo cavity, endosperm, absence of embryo or larva. The abnormalities found in the embryo were mainly observed as wrinkled or dried. Consequently, seed was considered abnormal if one or both structures (embryo cavity and endosperm) were deformed. To have a validation of this technique, a sample of seeds after X-ray, was opened and checked under the stereoscope to confirm the presence of embryo.

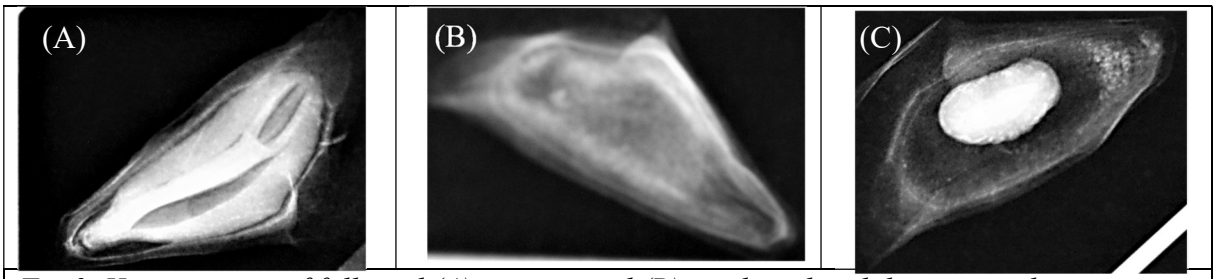



*Fig 3. X-ray images of full seed (A); empty seed (B); and seed with larvea inside*

### *3.3. Viability and germination assays for seeds and zygotic embryos*

Mature seeds from cones of *A. nebrodensis* trees (Nº 6, 7, 8, 10, 12, 13, 19, 21, 22 27) were maintained at 4 °C for 6 months. TTC and in vitro germination test were applied on seeds and zygotic embryos to evaluate the viability and germination before to begin the conservation experiments.

#### 3.3.1. Seed viability test by tetrazolium (TTC)

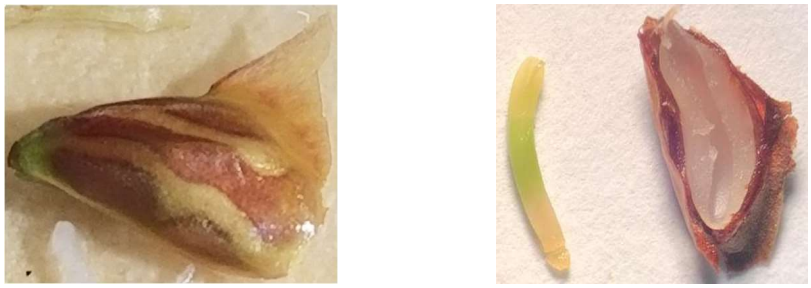
The tetrazolium (2,3,5-tryphenyl tetrazolium chloride, TTC) test to assess seed viability. Whole full seeds were soaked in tap water for 24 h and then seed coats were cut at two sides (Fig. 4A) to allow the penetration of TTC solution. These seeds were completely immersed in 0.1% (w/v) TTC in 50 mM Tris-HCl buffer (pH 7.6) for 24 h in darkness at 30 °C. After this treatment, the seeds were washed twice with distilled water and placed in petri dishes with moist filter paper. The development of red color (Fig. 4B) was confirmed by the cut longitudinally through endosperm, expose embryo, and remove seed coat. The color of zygotic embryo was the main indicator of whether the seed was alive or not (Fig. 4C).


(A) (B) (C)

*Fig. 4. 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).*

3.3.2. In vitro germination

For the sterilization, under laminar flow, full mature seeds were treated with 70% ethyl alcohol for 5 min, and rinsed with sterile distilled water 3 times. Then treated with 20% v/v sodium hypochlorite with 3 ml of Tween 20% solution for 20 min, and rinsed with sterile water 3 times. After sterilization, the seeds were imbibed in water for 48h under sterile condition (Fig. 5A) before the extraction of zygotic embryos (Fig. 5B).



(A) (B)

*Fig. 5. Imbibed seed (A); embryo excised from A. nebrodensis seeds (B)*

Excised zygotic embryos were cultivated *in vitro* to test the germination. To select the appropriate germination medium, two media were tested: MS medium (Murashige and Skoog 1962) hormone free (MS-H) and MS supplemented with 0.5 mg $L^{-1}$ $GA_3$ (MS + $GA_3$). Every two weeks of starting the in vitro culture, the germination rate was calculated according to the formula:

*Germination rate = (number of embryos germinated / number of embryos cultivated) * 100.*

In Table 1 are reported the embryo germination rate of each tree. In MS-H medium the range germination rate of embryos was of 66-100%, while in MS + $GA_3$ the embryos from seeds of trees n. 22 and 27 showed nil germination. The results showed a better percentage of germination in the MS medium hormone free (Fig. 6).

Table 1. Zygotic embryo germination rate after 2 weeks in vitro culture on two different media

| Tree Nº | Germination rate (%) in MS-H | Germination rate (%) in MS+$GA_3$ |
|---|---|---|
| 6 | 66.67 | 83% |
| 7 | 100 | 100 |
| 8 | 70 | 60 |
| 10 | 100 | 100 |
| 12 | 100 | 100 |
| 13 | 75 | 100 |
| 19 | 100 | 100 |
| 21 | 100 | 100 |
| 22 | 100 | 0 |
| 27 | 100 | 0 |

(A)

(B)

*Fig. 6. Embryos germination after 2 weeks (tree n°7) (A) and after 4 weeks (tree n°10) on MS-H culture media (B).*

### *3.4. Seed and zygotic embryo conservation*

#### 3.4.1. Seed conservation at low temperature (-18°C)

Following the international standard for long-term seed conservation (FAO/IPGRI 1994), the storage at or below a temperature −18°C, with seed moisture content (MC, 3 -7%) were considered. A part of seeds *A. nebrodensis* collected in 2020 were used to conservation experiment. The seeds moisture content was evaluated (Table. 2) and then the seeds were stored in freezer at -18°C. After 6, 9, 12 months of storage re-test germination and viability tests (TTC and in vitro germination) were conducted. Both assays demonstrated good results for each evaluated period.

Table 2. Moisture content of seeds collected from *A. nebrodensis* trees

| Tree N° | MC% | Seeds number | Quantity (g) |
|---|---|---|---|
| 6 | 6.76 | 334 | 19.5 |
| 7 | 5.65 | 458 | 20.6 |
| 8 | 6.25 | 784 | 36.2 |
| 10 | 5.56 | 3200 | 75.52 |
| 12 | 5.71 | 531 | 28.20 |
| 13 | 8.18 | 344 | 17.01 |
| 17 | 5.83 | 42 | 3.3 |
| 19 | 7.34 | - | - |
| 21 | 6.43 | 686 | 44.78 |
| 22 | 5.56 | 598 | 25.4 |
| 27 | 6.16 | 590 | 25.9 |

- Preparation of samples for permanent collocation in seed-bank

To ensure the storage of high quality seeds, only full seeds with healthy and undamaged embryo were counted, weighted and placed in labeled jars (DAGKLAR Jar, clear glass / stainless steel 0.4 l) containing all the associated information; location of seed bank: Museo MAN, species: *Abies nebrodensis,* plant number: 6, 7, 8,….27, collection year: 2020, quantity (gr), seeds number, starting date of conservation: 15/11/2021 (Fig. 7A). Jars were prepared in CNR-IBE and transported to the seed bank located in MAN (Museum of *Abies nebrodensis) in* Polizzi Generosa (PA), where they are stored in freeze chambers (-18°C) (Fig. 7B).


*Fig. 7. Jar labeled with seed sample information*

*Fig. 7. Seed-bank of A. nebrodensis with some seed samples*

3.4.2. Excised zygotic embryos conservation at ultra-low temperature (-196°C)

Zygotic embryos extracted from the sterilized mature seeds were used for the cryopreservation. The MC of zygotic embryos (tree n°10 seeds) determined by the Moisture Analyzer was 8.91%. To carry out the experiment, zygotic embryos were divided into two groups, one treated with plant vitrification solution (+PVS2) and other without (-PVS2). PVS2 is cryoprotective solution consisted of 30 % glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide (DMSO) in MS, 0.4 M Sucrose (Fig. 8A). The cryovials containing embryos inside the cryobox were immersed in LN. Thereafter, the cryobox was taken out from the liquid nitrogen (Fig. 8B) and thawed in a water bath (40 °C) for 1 minute.

(A) (B)

*Fig. 8. Cryovial containing embryos in PVS2 (A); taking off the cryobox from LN.*

All the cryovials (+PVS2/-PVS2) were placed under the laminar flow and PVS2 solution was discharged and replaced with liquid MS medium containing 1.2 M sucrose for 20 min (washing solution). After cryopreservation, zygotic embryos treated with or without PVS2 were subjected to a test of viability (TTC) and in vitro germination (MS-H medium).

Germination of zygotic embryos treated with PVS2 solution (Fig. 9A) were similar to those stored without PVS2 (Fig. 9B). Indeed, an optimal germination percentage of 90-95% was recorded regardless of the presence of PVS2. Also, all the embryos stained with TTC showed a completely red colour after recovery from LN (Fig. 9C). Hence, the results show the potential of the cryogenic technology for the preservation of *A. nebrodensis* zygotic embryos.


(A) (B) (C)

*Fig. 9. Germinated embryo after cryopreservation: without PVS2 (A) or with PVS2 (B) treatment and viable embryos stained with TTC (C).*

3.4.3. Seed conservation at ultra-low temperature (-196°C; cryopreservation)

To evaluate the possibility of the cryopreservation of entire seeds, a sample of seeds from tree Nº 10 (MC %= 5.56) was inserted in cryovials (Fig. 10A) (Fig. 10B). The cryobox with cryovials is immersed directly into LN (without any cryoprotective treatment). After cryopreservation (Fig. 10C), samples were thawed in a water bath at 40 °C for 2 min (Fig. 10D).

(A) (B) (C) (D)

*Fig. 10. Cryovials with seeds (A); Cryobox with cryovials (B); Direct immersion of cryobox in LN (C); Thawing in water bath (D).*

To test the validation of the procedure after seeds cryopreservation, TTC and In vitro germination tests were applied.

The cryopreserved seeds after the TTC test were placed on moist filter paper for viability evaluation. Indeed, different types of cuts were transversally (Fig. 11B) and longitudinally (Fig. 11C) performed to demonstrate the red staining of seeds. According to the previous results on in vitro germination of seeds, MS hormone free medium was selected to test the viability of embryos extracted from cryopreserved seeds. Therefore, excised embryos were cultured *in vitro* on MS-H medium supplemented with 2% sucrose and 0.7% agar.

The cryopreserved seeds after LN treatment showed 85-90% of viability through TTC estimation as well as in vitro germination. Seeds were considered viable when they showed: embryo (Fig. 11A) and the endosperm with embryo (Fig. 11C) in intense red color. Also, the in vitro germination confirmed the storability of *A. nebrodensis* seeds in LN for a long-term storage (Fig. 11D).

(A) (B) (C) (D)

*Fig. 11. Embryo (A); seed cut transversally (B) and longitudinally (C) completely stained in red color; germination in gelled medium (D).*

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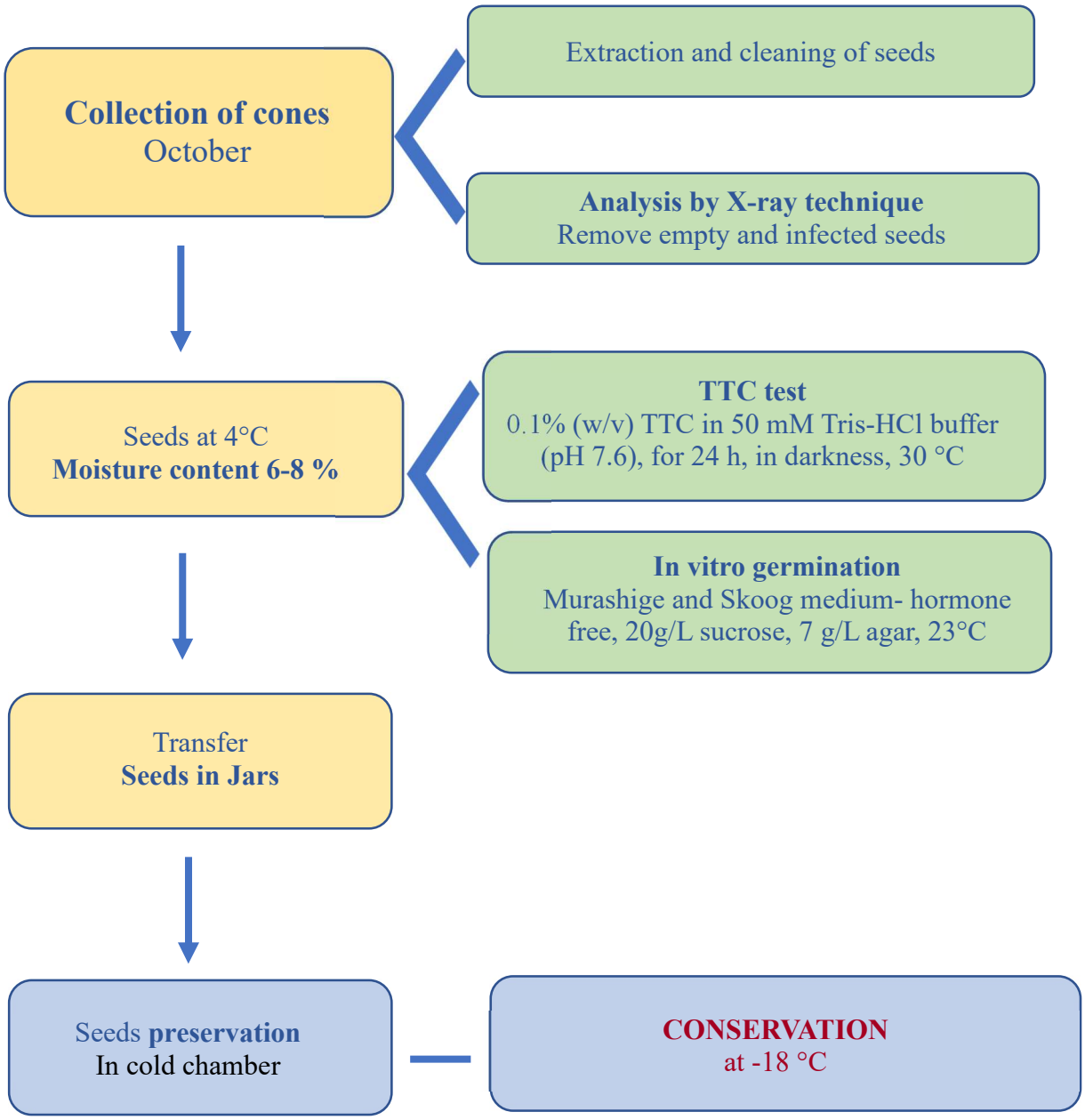
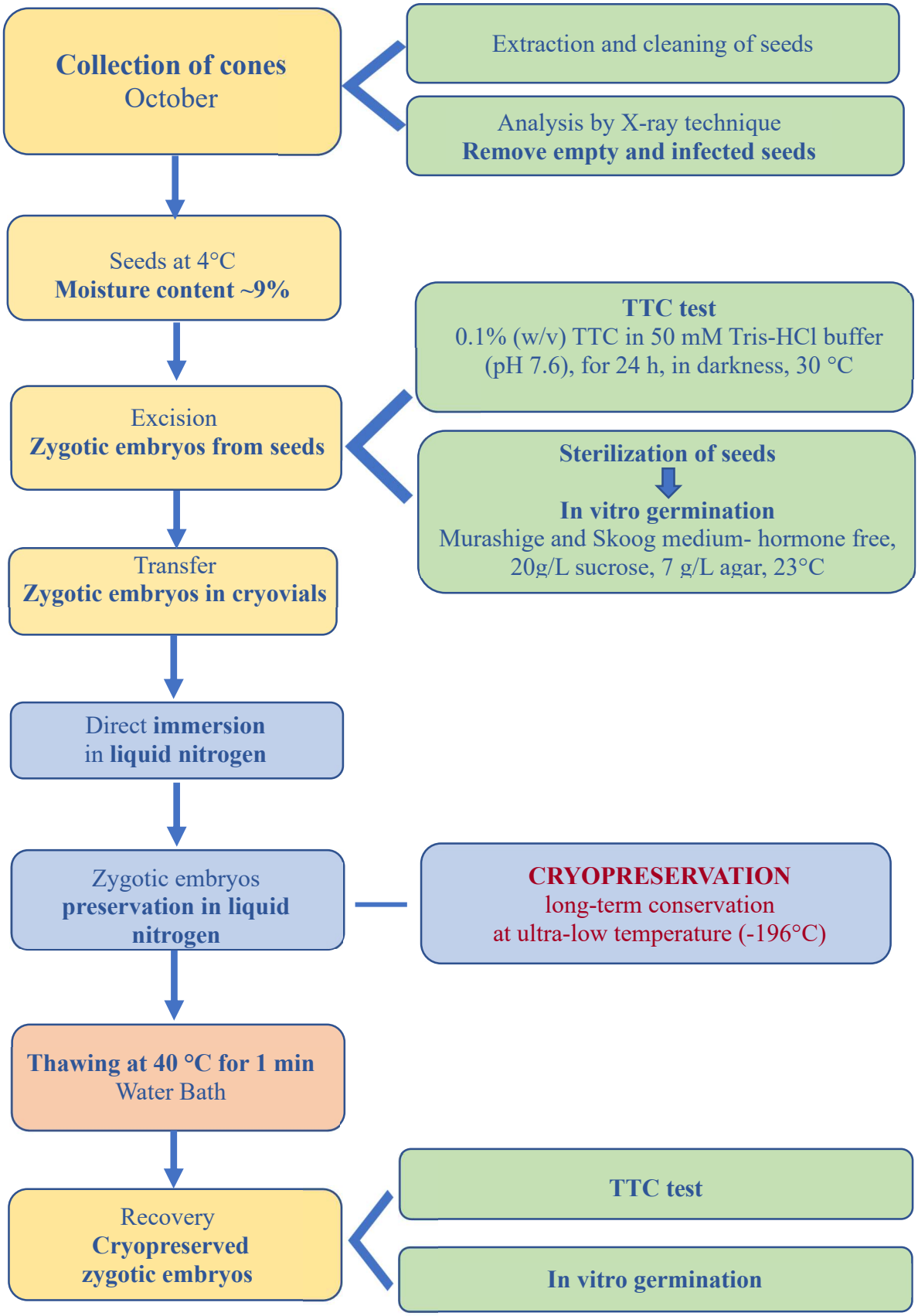
## 4. Final Protocol for *A. nebrodensis* conservation in seed bank (-18°C)



## 5. Final Protocol for *A. nebrodensis* zygotic embryos conservation in cryobank (-196°C)



## 6. Final Protocol for *A. nebrodensis* seeds conservation in cryobank (-196°C)