Actions A.1 and C.5

Improvement of the conservation at low and cryogenic temperatures of selected tissues and organs → constitution of a seed bank and a cryobank for seeds, pollen, isolated embryos and embryogenic callus lines.

Maurizio Lambardi, Carla Benelli

CNR, IBE-Institute of BioEconomy, Sesto Fiorentino (Firenze)
Maurizio Lambardi, Carla Benelli, Tolga Izgu, Waed Tarraf

Università degli Studi di Palermo
Maria Antonietta Germanà, Rosario Schicchi, Jouini Nourhen

Ente Parco delle Madonie
Peppuccio Bonomo

C5 – Constitution of a seed bank and a cryobank for the long-term conservation of seeds, pollen, isolated embryos and embryogenic callus lines of A. nebrodensi

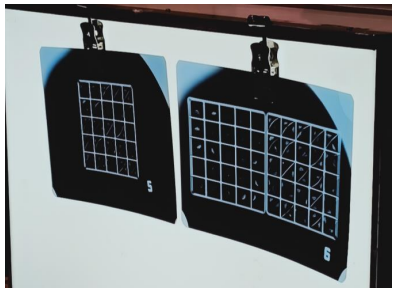
Constitution of the structures for the seed bank and the cryobank (i.e., designation of the rooms / spaces dedicated to aforesaid banks, assurances of the continuous energy and liquid nitrogen supply) → SEED BANK & CRYOBANK operative	**21/7/2023**

Inaugurazione della Banca del Seme e della Criobanca del MAN

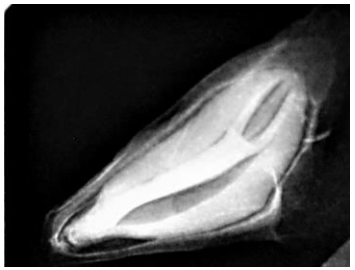
SEED BANK → Conservation of mature seeds

Aim: conservation of seeds at -18°C (100 g per tree)

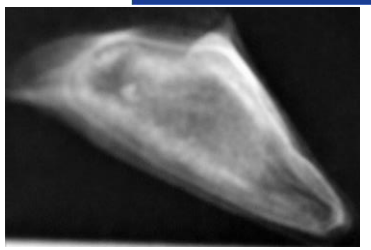
Tree n°	Full seeds (%) 2020	Full seeds (%) 2022
6	13	11
7	54	18
8	37	18
10	36	37
12	25	12
13	15	14
17	43	16
19	0	0
21	44	32
22	31	20
23	28	31
25	0	2
27	19	11



Checking of the seeds on X-Ray Film Viewer Screen



Full seed



Empty seed

SEED COLLECTIONS

2020

Max: 54% (tree n°7)
Min: 0% (tree n°19)
Average (-tree n°19): 31,7%

2021: no seed production

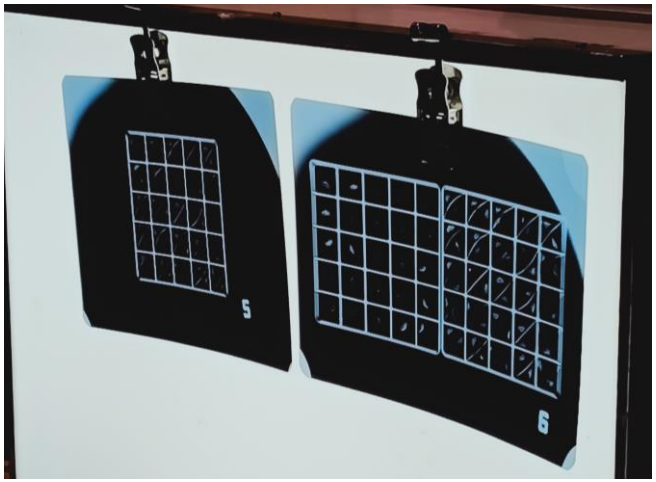
2022

Max: 37% (tree n°10)
Min: 0% (tree n°19)
Average (-tree n°19): 18,5%

2023: no seed production

SEED BANK → Conservation of mature seeds

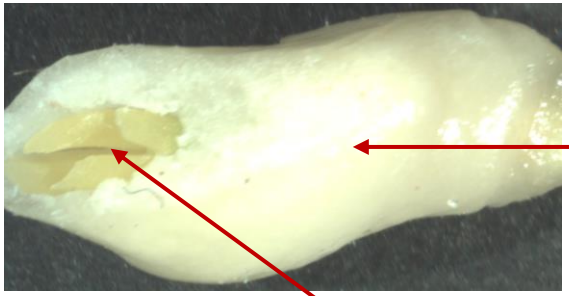
Aim: conservation of seeds at -18°C (100 g per tree)



Checking of the seeds on X-Ray Film Viewer Screen

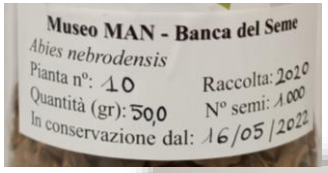
fs

Empty seeds and full seeds



***Validation:** opening a sample of seeds under the stereoscope*

La Banca del Seme (SEED BANK) del MAN



Albero n°	2020		2022	
	n° semi conservati	Quantità (gr)	n° semi conservati	Quantità (gr)
6	334	19.5	134	8.2
7	458	20.6	-	-
8	784	36.2	181	13.0
10	1000	50.0	193	13.8
12	531	28.2	118	7.1
13	344	17.0	471	22.0
17	42	3.3	-	-
21	686	44.8	323	18.7
22	598	25.4	285	11.1
23	-	-	99	4.5
25	-	-	3	0.2
27	200	9.2	156	8.1

A.1 project deliverable

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

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

Collection of cones
October

- Extraction and cleaning of seeds
- **Analysis by X-ray technique**
  Remove empty and infected seeds

Seeds at 4°C
Moisture content 6-8 %

- **TTC test**
  0.1% (w/v) TTC in 50 mM Tris-HCl buffer (pH 7.6), for 24 h, in darkness, 30 °C
- **In vitro germination**
  Murashige and Skoog medium- hormone free, 20g/L sucrose, 7 g/L agar, 23°C

Transfer
Seeds in Jars

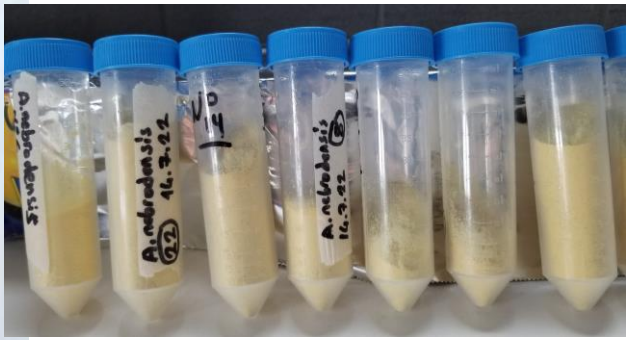
Seeds **preservation**
In cold chamber

CONSERVATION
at -18 °C

CRYOBANK → Cryopreservation of pollen

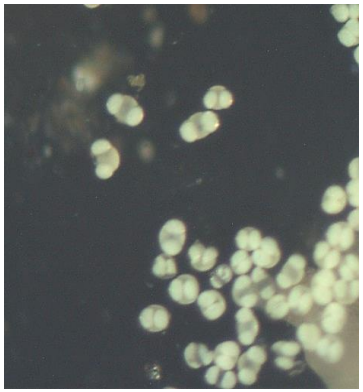
Pollen collection

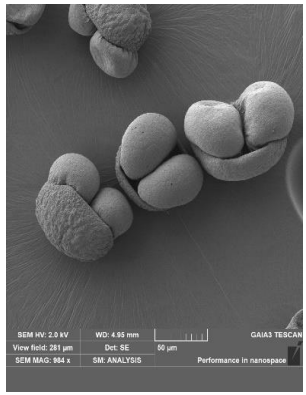
Collection 2022



Pollen sieving

(A) (B) (C)

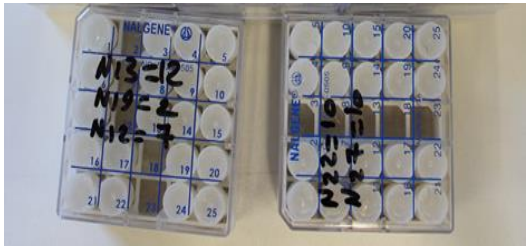


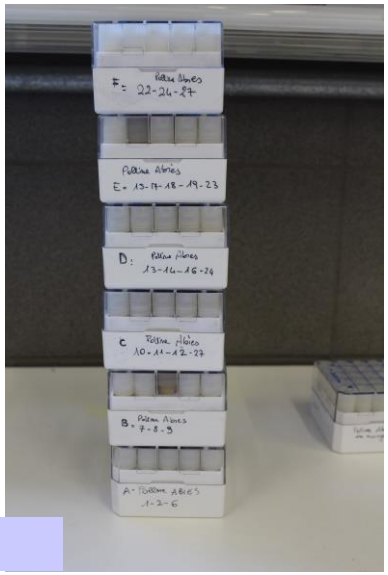


A. nebrodensis pollen under:
stereomicroscope (A)
optical microscope (B)
Environmental Scanning Electron Microscope (ESEM; C)

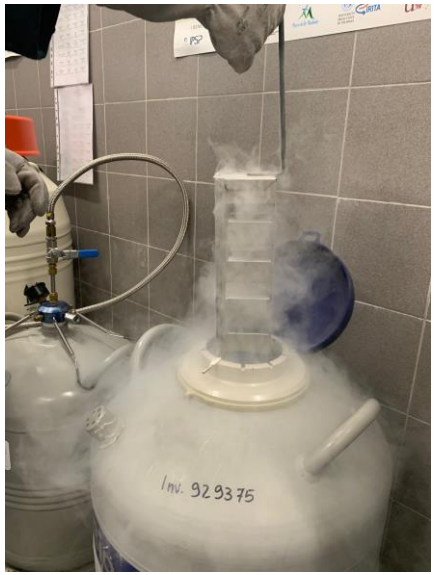
Moisture content evaluation

Pollen in cryovials





Pollen in cryobox



Liquid nitrogen (-196°C)

Viability and Germinability percentage of pollen grains

Plant N°	MC %	Viable	Germinable
1	7.48	78,83	84,72
2	7.84	9,17	8,15
6	11.29	90,00	88,89
7	9.17	88,89	90,00
8	10.19	98,89	91,11
9	9.52	79,17	87,78
10	8.91	82,50	98,61
11	9.84	90,56	93,89
12	8.65	46,67	92,22
13	9.35	86,67	89,44
14	8.40	67,22	99,44
15	10.32	63,61	99,72
16	8.74	90,28	90,83
17	9.62	93,61	85,83
18	7.76	96,39	86,58
19	6.86	26,89	23,89
21	6.78	11,46	22,08
22	10.89	33,06	91,67
23	10.06	98,89	98,06
24	8.74	94,44	98,89
27	10.54	73,08	90,83
29	9.40	11,12	8,69

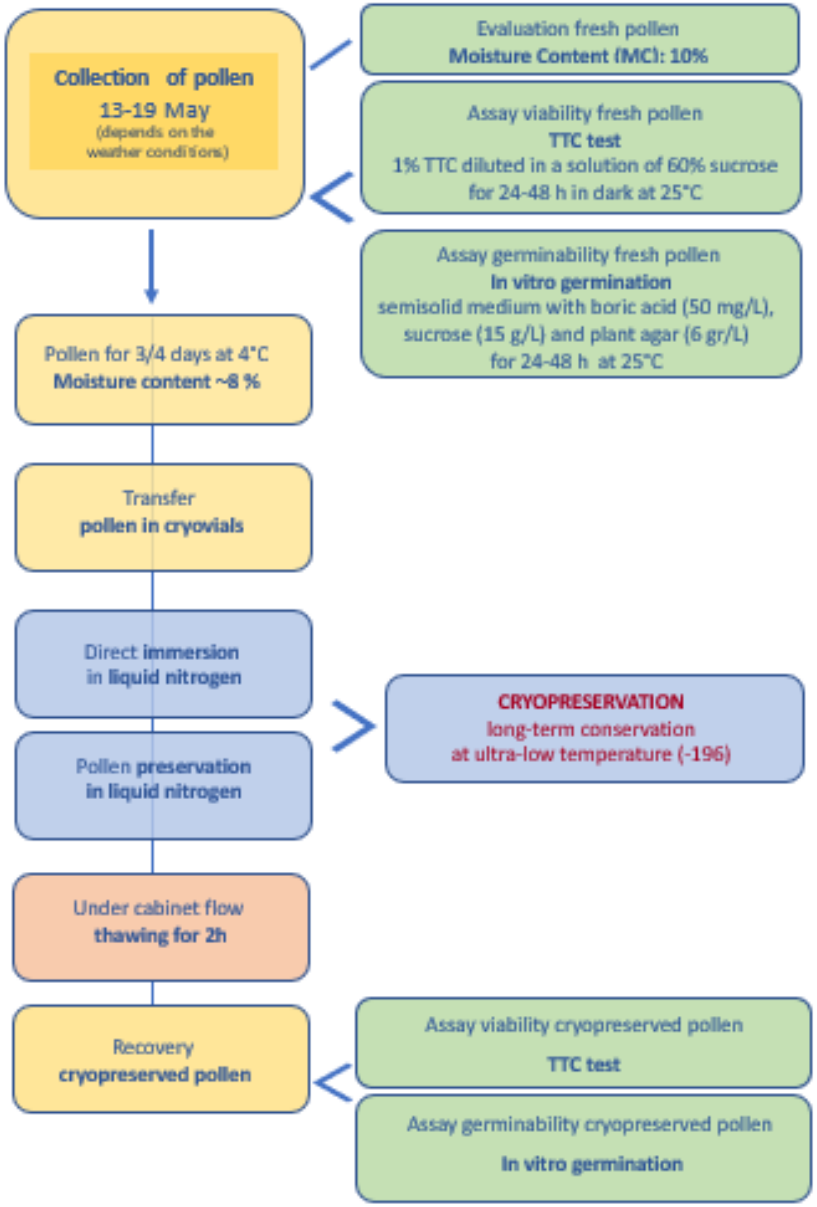
Il polline di 23 alberi, raccolto negli anni 2020 e 2023, è stato mantenuto a -18°C presso l'Università di Palermo.

Trasferito nella criobanca del museo MAN il 20 luglio 2023.

A.1 project deliverable

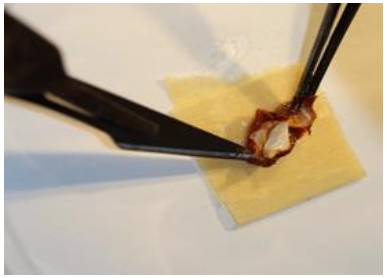
Report of a complete protocol of long-term conservation of A. nebrodensis pollens at ultra-low (i.e., cryogenic) temperature

Protocol for *A. nebrodensis* pollen conservation in cryobank (-196°C)



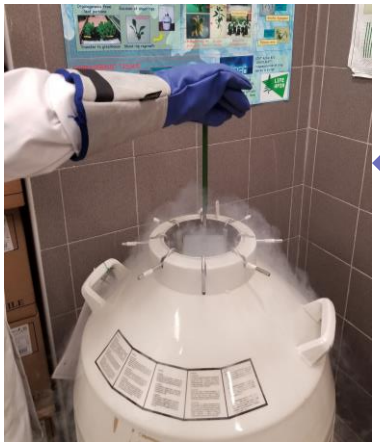
CRYOBANK → Cryopreservation of excised zygotic embryos

Sterilized seeds



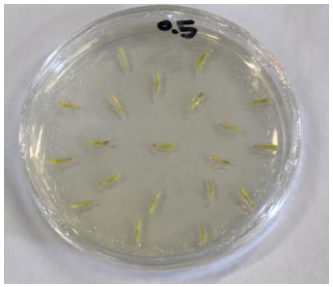
Excision of zygotic embryos from full seeds selected at X-Ray

Cryovial with embryos in PVS2 solution



Liquid nitrogen (-196°C)

Thawing in water bath for 1 min at 40°C

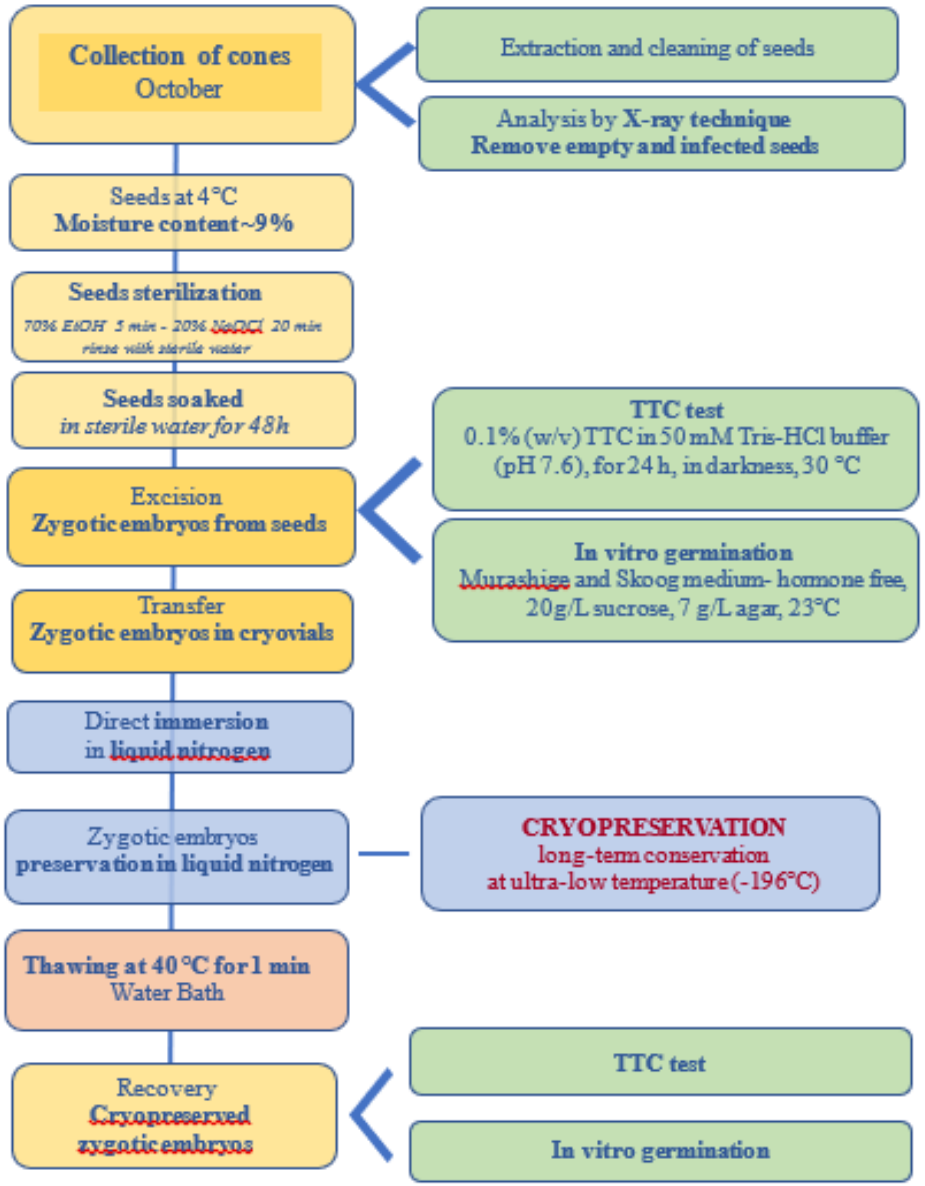


Recovery cryopreserved embryos

A.1 project deliverable

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

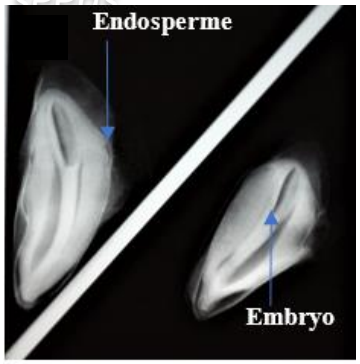
Protocol for *A. nebrodensis* zygotic embryos conservation in cryobank (-196°C)



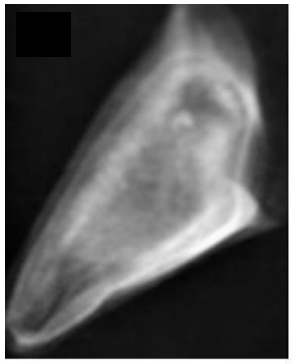
CRYOBANK → Embryogenic callus lines from mature embryos

Mature cone and seeds (collection, late September)

X-Ray analysis to select full seeds



Full seed



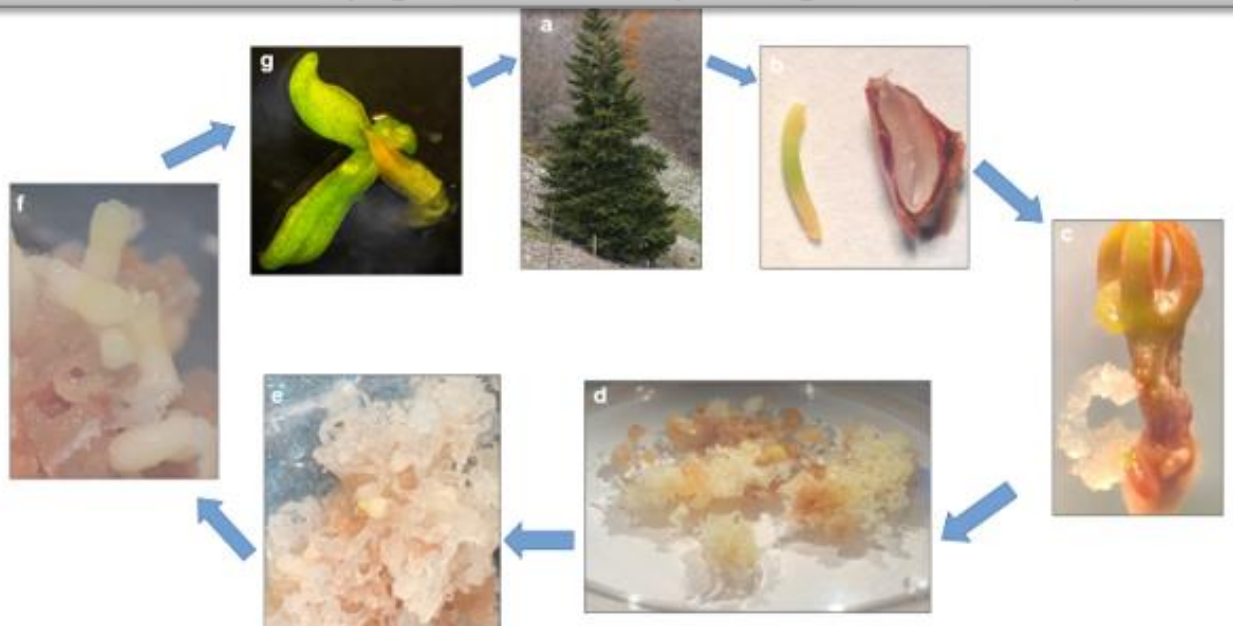
Empty seed

Seed ready to be opened for embryo extraction

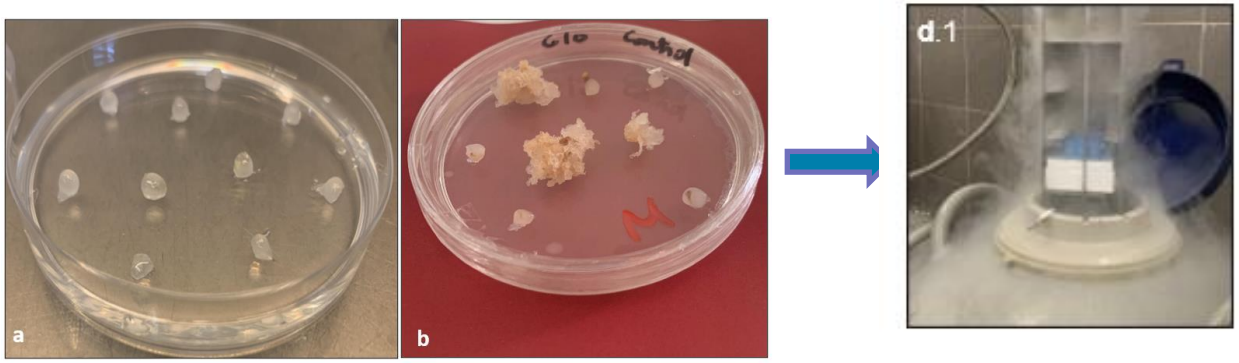
Mature embryos on gelled medium for embryogenic callus induction

Excised mature zygotic embryos

CRYOBANK → Cryopreservation by encapsulation-dehydration of EC samples



Synthetic seeds



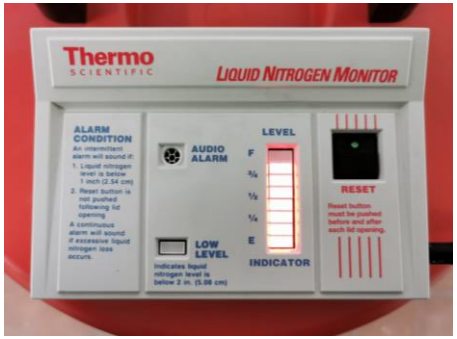
Le linee embriogeniche di 8 alberi sono state prima trasferite dall' Università di Palermo al CNR-IBE per la preparazione di semi sintetici.

I semi sintetici delle 8 linee sono stati trasferiti nella criobanca del museo MAN il 20 luglio 2023.

Cronologia della costituzione della criobanca presso il MAN e aspetti tecnici di gestione

28 giugno 2023

20 luglio 2023: Ricarica e implementazione della criobanca



3 settimane: 1 tacca (circa 15 lt di AL consumati)

21 luglio 2023: Inaugurazione ufficiale della Banca del Seme e della Criobanca del MAN

Polline in Criobanca a Polizzi Generosa		
Albero N°	N° criovials Anno 2020	N° criovials Anno 2022
1	1	5
2	1	2
6	1	10
7	1	2
8	1	14
9	1	20
10	1	7
11	1	10
12	1	10
13	1	14
14	1	12
15	1	15
16	1	3
17	1	6
18		4
19	1	3
21	1	1
22	1	18
23	1	2
24	1	5
25	1	0
27	1	9
29		1

Embrioni zigotici in Criobanca a Polizzi Generosa		
Albero N°	N° Criovials Anno 2020	N° totale embrioni
6	20	100
8	21	105
10	18	90
13	15	75
21	18	90
22	18	90
27	16	80

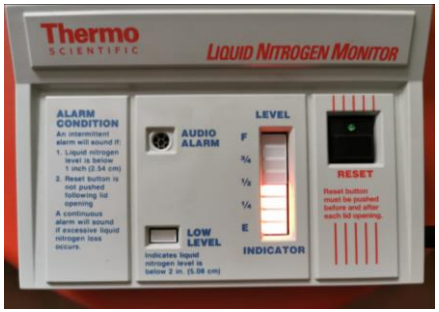
* 5 embrioni in criovial

Callo embriogenico in Criobanca a Polizzi Generosa		
Albero N°	N° criovials 2020 maturi	N° totale Capsule
7	11	44
8	11	44
10	11	44
11	X	X
15	X	X
16	X	X
21	11	44
22	10	40

4 capsule / criovial; X =da aggiungere

18 ottobre 2023: Prima ricarica di azoto liquido

20 luglio 2023



12 ottobre 2023

Ringraziamento speciale a Lorenzo Sausa

83 giorni = c.a 12 settimane:
consumo di circa 60 litri di AL

1 ricarica di 60 lt ogni c.a 3 mesi

Costo a ricarica: € 450 (*300 trasposto+ € 2,5/lt*) ogni 3 mesi c.a
Costo annuo: € 1.800 c.a

Le 11 forniture LIFE4FIR termineranno a luglio 2025

In Cryobank:

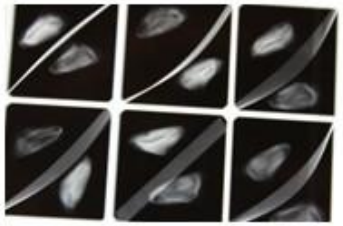
- ✓ Excised embryos

- ✓ Pollen

- ✓ Embryogenic callus

In Seedbank:

- ✓ Seeds



LIFE18 NAT/IT/000164 - C1a

A1's PROJECT DELIVERABLE PRODUCTS

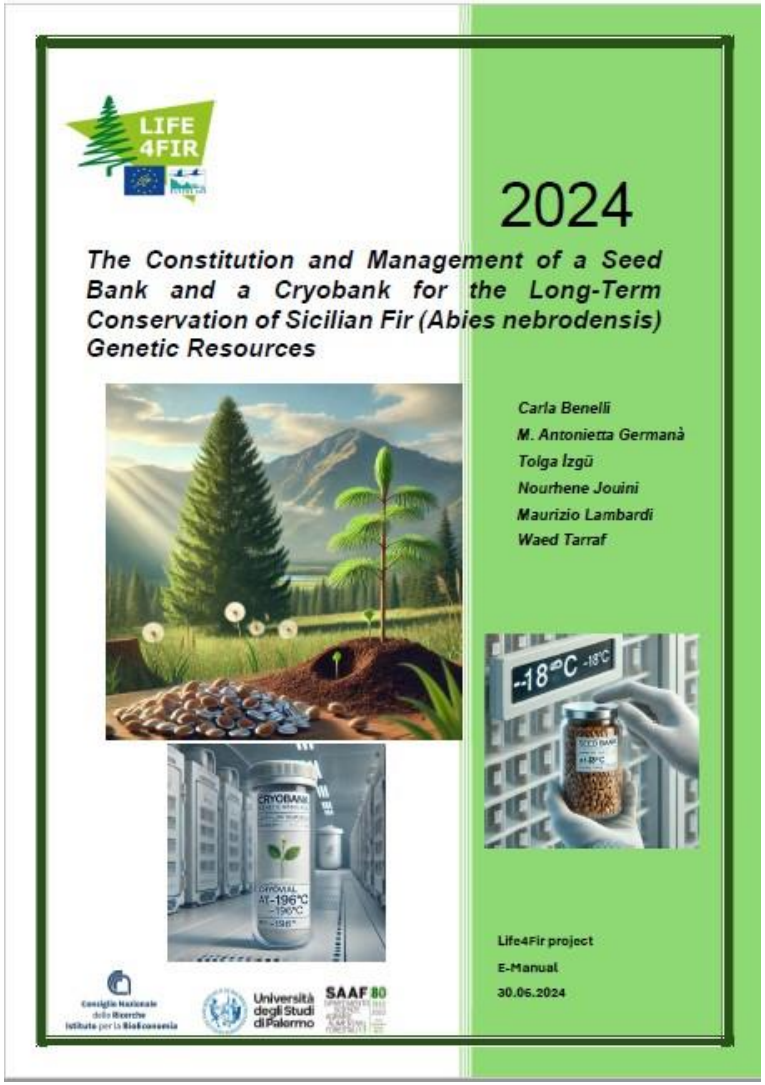
Deliverable name	Deadline	
Report on optimized protocols for the reproduction of A. nebrodensis trees by seed and grafting propagation	12/2020	→ Delivered
Report of a complete protocol for A. nebrodensis seed and excised zygotic embryo conservation at low (-18°C) and cryogenic (-196°C) temperatures, respectively	12/2020	→ Delivered
Report of a complete protocol of long-term conservation of A. nebrodensis pollens at ultra-low (i.e., cryogenic) temperature	12/2020	→ Delivered

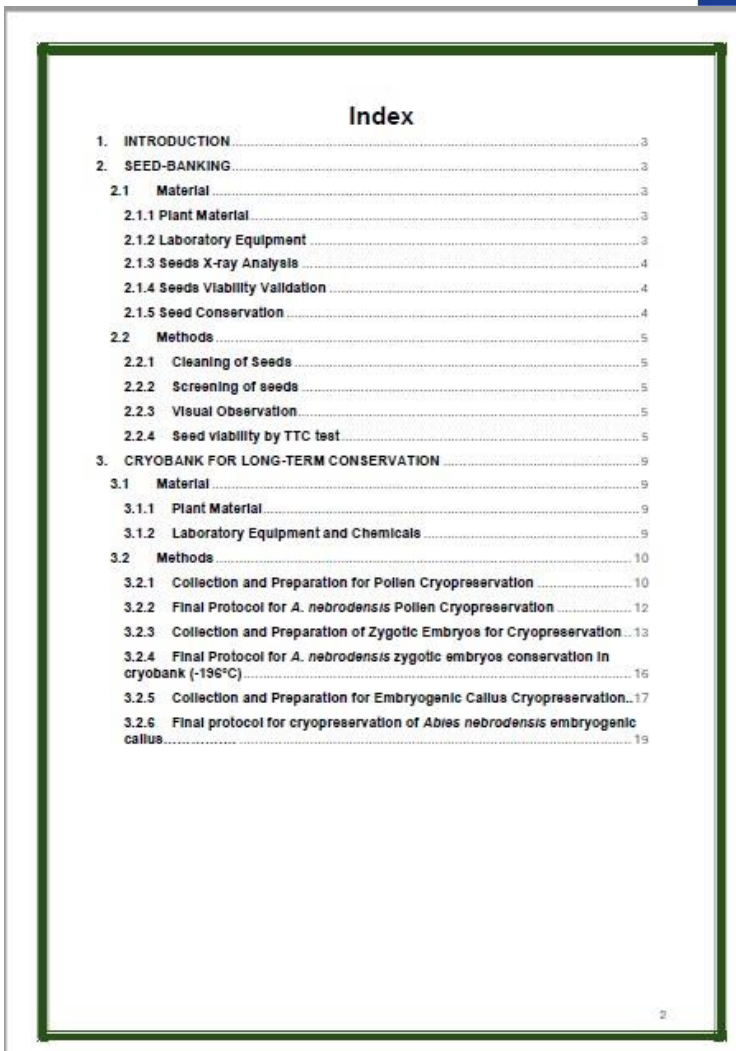
LIFE18 NAT/IT/000164 - C1c

C5's PROJECT DELIVERABLE PRODUCTS

Deliverable name	Deadline	
Report on the seed- and cryobanks constitution	07/2021	→ Delivered
Database of the pollen/excised embryos/somatic embryogenesis samples in the cryobanks reported in the website	01/2023	→ Delivered
E-manual on the website for downloading, containing practical information on the constitution and management of seed and cryobank for the long-term conservation of fir genetic resources	04/2022	→ Delivered
Report of a complete protocol of somatic embryogenesis and cryopreservation of proembryonic masses of A. nebrodensis	12/2021	→ Delivered
Database of the seed samples in the bank reported in the website	03/2023	→ Delivered

E-manual





Index

Tarraf W., Izgu T., Benelli C., Lambardi M., Germanà M.A., Jouini N. (2021). Biotechnology for the conservation of critically endangered plant species: the example of *Abies nebrodensis*, a relict conifer of Sicily. In 10th International Molecular Biology and Biotechnology Congress (MolBiotech 2021). p. 40. Turkey, 4-8 October 2021.

Tarraf W., Izgu T., Benelli C., Lambardi M., Germanà M.A., Jouini N. (2021). Studi preliminari per lo sviluppo di procedure idonee alla conservazione a lungo termine di *Abies nebrodensis*. In XIII Convegno Nazionale sulla Biodiversità "Agricoltura, Ambiente e Salute". p. 241. Foggia-Italia, 7, 8 e 9 settembre 2021.

Tarraf W., Izgu T., Jouini N. (2022). Strategies for the conservation by biotechnological approaches of *Abies nebrodensis*, a relict conifer of Sicily. Acta Hort.

Plant Cell, Tissue and Organ Culture (PCTOC)
https://doi.org/10.1007/s11240-022-02415-0

ORIGINAL ARTICLE

Somatic embryogenesis in *Abies nebrodensis*, an endangered Sicilian fir

Nourhene Jouini[1] · Emna Yahyaoui[1] · Waed Tarraf[2] · Tolga Izgü[2] · Carla Benelli[2] · Maurizio Lambardi[2] · Maria Antonietta Germanà[1]

Received: 13 September 2022 / Accepted: 4 November 2022
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Abstract
Somatic embryogenesis, as a promising biotechnological tool for many conifer trees, has never been applied for the *Abies nebrodensis* species. Although all the encouraging results previously obtained by the EU LIFE (European LIFE program) funded projects in over ten years, the critically endangered Sicilian fir remains alarmingly close to extinction. In this study, we reported the first protocol of somatic embryogenesis obtained from mature zygotic embryos of the *Abies nebrodensis*. Seeds from *Abies* adult trees with specific identification numbers (IN) were collected and full seeds were identified by X-ray. Different experiments were carried out for callus initiation, from both zygotic immature and mature embryos, testing different culture media. The immature embryos did not give embryogenic tissue (ET). Embryogenic callus (EC) was successfully induced from mature embryos with variable frequencies (0–40%). Schenk and Hilderbrandt (SH) was the most suitable initiation medium where the obtained callus initiation rate reached up to 40% for IN7 (first experiment). 6-benzylaminopurine (BAP) showed to be essential to induce EC (second experiment). IN8 presented the highest callus initiation rate (40%) among all tested donor trees, whereas IN13 recorded the lowest rate with 4% (third experiment). ET maturation from each singular embryo of IN7, IN8, IN10 and IN21 was successfully achieved in SH medium containing 37,83 µM abscisic acid (ABA), 8% of polyethylene glycol (PEG-4000) and 4% maltose. The encapsulation technology was assessed on the obtained ET and its proliferation was observed after encapsulation.

Key message
A protocol for somatic embryogenesis from mature embryos of the *Abies nebrodensis* was achieved.

Keywords Nebrodi fir · In vitro culture · Biodiversity · Callus induction · Cryoconservation · Synthetic seeds

plants

MDPI

Article

Long-Term Conservation for the Safeguard of *Abies nebrodensis*: An Endemic and Endangered Species of Sicily

Carla Benelli [1], Waed Tarraf [1,*], Tolga İzgü [1], Monica Anichini [1], Cecilia Faraloni [1], Maria Cristina Salvatici [2], Nourhene Jouini [3], Maria Antonietta Germanà [3], Roberto Danti [4] and Maurizio Lambardi [1]

[1] Institute for BioEconomy (IBE), National Research Council (CNR), Via Madonna del Piano 10, 50019 Sesto Fiorentino, Florence, Italy; carla.benelli@ibe.cnr.it (C.B.); tolga.izgu@ibe.cnr.it (T.İ.); monica.anichini@ibe.cnr.it (M.A.); cecilia.faraloni@ibe.cnr.it (C.F.); maurizio.lambardi@ibe.cnr.it (M.L.)
[2] Institute of Chemistry of Organometallic Compounds (ICCOM)-Electron Microscopy Centre (Ce.M.E.), National Research Council (CNR), Via Madonna del Piano 10, 50019 Sesto Fiorentino, Florence, Italy; salvatici@ceme.fi.cnr.it
[3] Department of Agricultural, Food and Forestry Sciences, University of Palermo, 90128 Palermo, Italy; nourhene.jouini@unipa.it (N.J.); mariaantonietta.germana@unipa.it (M.A.G.)
[4] Institute for Sustainable Plant Protection (IPSP), National Research Council (CNR), Via Madonna del Piano 10, 50019 Sesto Fiorentino, Florence, Italy; roberto.danti@ipsp.cnr.it

After Life4Fir.....

- Proseguire nella collaborazione con il Parco delle Madonie, l'Università di Palermo e il Municipio di Polizzi Generosa nel comune intento di completare negli anni l'implementazione della Banca del Seme e della Criobanca con semi es espianti dell'Abete delle Madonie
- Offrire un servizio di conservazione in Banca del Seme e in Criobanca per utenti interessati alla salvaguardia di altre conifere sottoposte ad erosione genetica
- Mettere l'esperienza maturata con il Life4Fir nella conservazione ex situ dell'Abete delle Madonie a disposizione per la creazione di strutture analoghe a quella realizzata nel MAN