Training School on Dormant Bud Cryopreservation

21-23 May 2024, Faenza, Italy

centro attività vivaistiche

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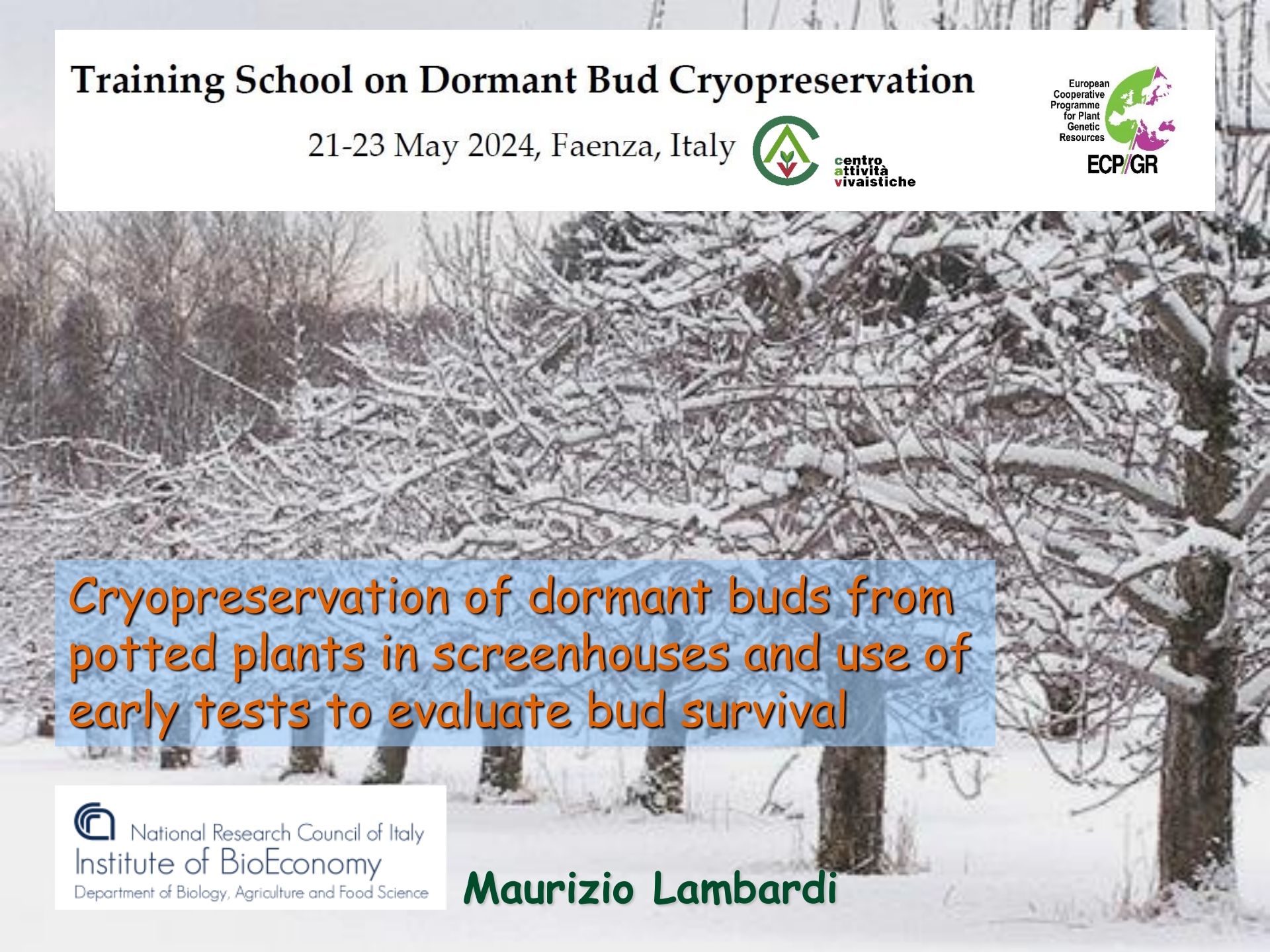


Cryopreservation of dormant buds from potted plants in screenhouses and use of early tests to evaluate bud survival

National Research Council of Italy
Institute of BioEconomy
Department of Biology, Agriculture and Food Science

Maurizio Lambardi


AXILLARY BUDS

Treatment with PVS2 solution

Encapsulation--dehydration

Droplet methods

Encapsulation--vitrification

D-, V-cryoplate

Dehydration

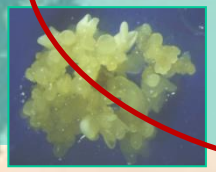
Dehydration + slow cooling (-1°C/h)

SEEDS AND EMBRYONIC AXES, POLLEN

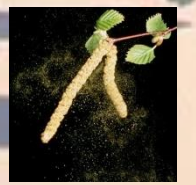
"Two-step" freezing (-1°C/min)

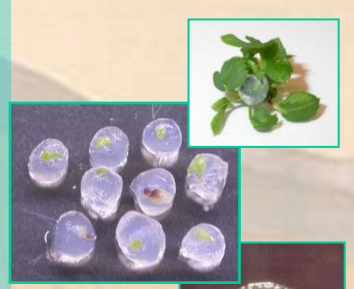
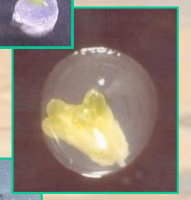
Dormant (winter) buds

CELL AND CALLUS CULTURES

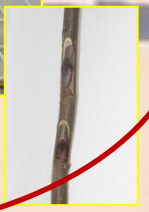











Lambardi e De Carlo, 2009. Italus Hortus.

«Dormant» or «winter» buds? The ambiguity of the term «dormant buds»……

- the technique is named «cryopreservation of dormant buds», referring to the fact the buds are **collected in winter**;
- but, in fruit growing, the term «dormant» refers to the **physiological status of a bud** that is not induced to sprout yet;
- hence, the «dormant bud» is the **bud of the current season**, used for grafting in summer (July) and that will sprout in the following spring;
- so, to avoid misunderstandings, it would be better to use the terminology «**cryopreservation of winter buds**».

Tissue culture-based technique: "vitrification" of shoot tips

Dormant bud-based cryopreservation technique

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Cryopreservation of dormant buds

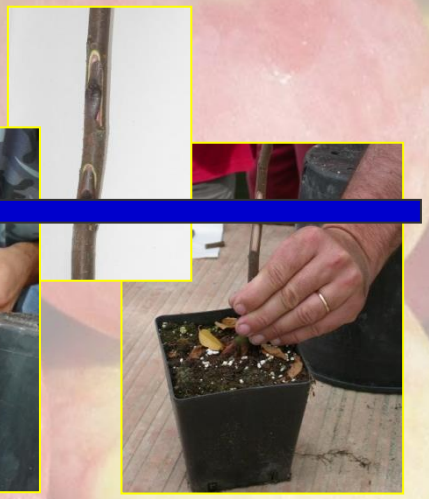
Towill L.E. e Ellis D.D., 2008. Cryopreservation of dormant buds. In: Reed B.M. (ed) Plant Cryopreservation. A Practical Guide. Springer, New York, pp. 421-442.

Winter collection of scions and cold hardening at -5°C (8 wks)

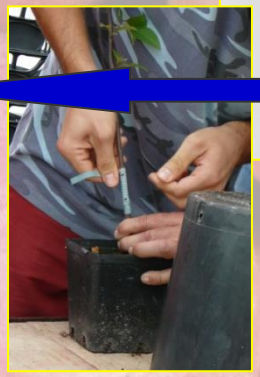
Uni-nodal segments ('micro-scions'), cold hardened at 4°C and dehydrated up to a MC of 35-26% (6-8 wks)

Slow cooling up to -30°C (-1°C/hour)

Thawing and rehydration in damp peat at 2°C (2 wks)



Chip budding



After 1-2 wks

After 6 wks

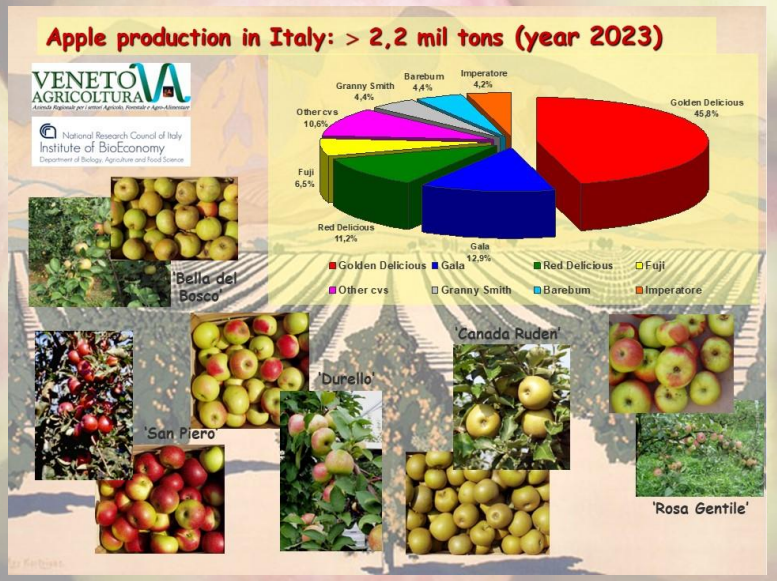
Rootstock: *Malus floribunda*, cv Radian

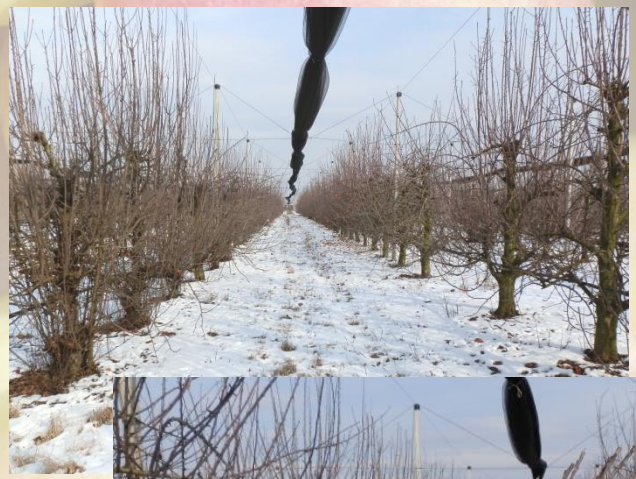
1. Scion collection and cold-hardening → from the field

Scion wood collected from plants in the field (mid-winter)

⇩

Scions wrapped in plastic bags and cold-hardened at -5°C (8 weeks)





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1. Scion collection and cold-hardening → from the screenhouse

C.A.V. ONGOING PROJECTS

Digital Droplet PCR

- canditate plants
- latent pathogens
- higher Sensitivity
- in house

Next Generation Sequencing NGS

- prescreening on candidate plants
- all viruses and viroids in one shot in outsorcing
- faster release of materials to nurseries

Cryopreservation of «pre-base» mother plants

- collaboration with CNR – IBE
- back-up copy of obsolete and low interest accessions
- apple, plum, cherry, pear

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From the field

scion sections were collected from vigorous 3- to 6-year-old trees of each accession at the end of a 72-h period when orchard ambient temperatures remained ≤0 °C . Of the 64 accessions studied, 51

Forsline te al., 1998. J. Amer. Soc. Hort. Science

From the screen-house

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"Pre-base" apple plants preserved in screen-house at the CAV.
Temperatures only occasionally drop below 0°C.

2. Scion sectioning and cold desiccation

⇩

Scions cut into 35-mm nodal sections, with one bud (micro-scions), and desiccated at - 5°C up to a MC of 25-30% (14-40 days)

Scion sectioning

Cold desiccation

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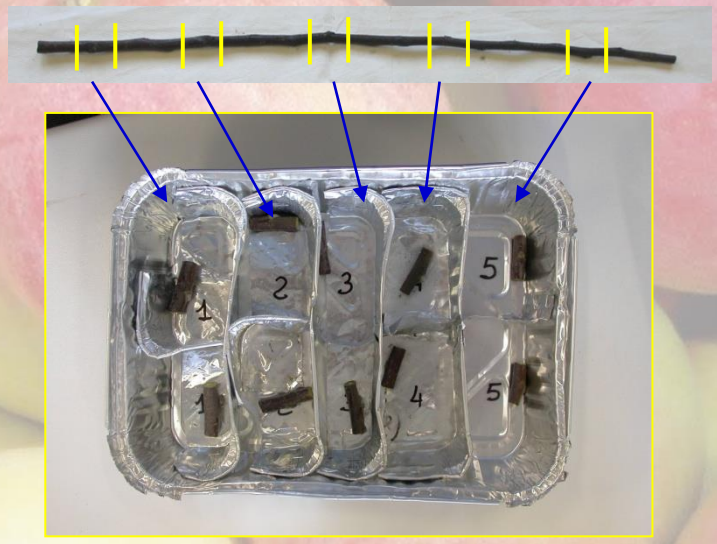
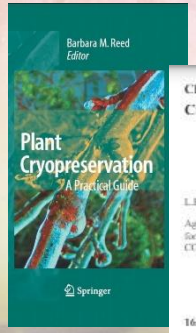
3. Moisture content determination → by weighting segment samples

⇩

Moisture content is determined every week (initially), then every 2-3 days by weighting a sample of 5 segments





Chapter 16

Cryopreservation of Dormant Buds

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16.1 General Status and Important Factors

Dormant vegetative buds from diverse species can be preserved using cryopreservation. Sakai (1960) provided one of the first studies showing that winter twigs of poplar (*Populus sieboldii*) and willow (*Salix koriyanagi*) could survive low temperatures if slowly cooled prior to immersion in liquid nitrogen. A later study demonstrated that this simple methodology was also applicable to twigs of several fruit species (Sakai and Nishiyama 1978). With rising interest in the preservation of genetic resources, methodologies were further developed for fruit, nut, forest and ornamental species that can cold acclimate. Although dormant buds from cold hardy herbaceous perennial species might also be useful for cryopreservation, there are few studies, with the exception of garlic, that addressed the use of cryopreservation to preserve dormant buds from herbaceous species. It should also be emphasized that in this chapter, we use the term "dormant" in a broad sense to include buds that are dormant due to either endogenous (endodormancy) or to a variety of environmental conditions (ecodormancy).

The methods for cryopreservation of dormant buds utilize techniques described for other systems, including controlled rate cooling, vitrification, and encapsulation dehydration. The main difference is that a dormant bud is used for these techniques as contrasted to an actively growing shoot tip.

421

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide*
© Springer 2008

$$MC = \frac{(\text{Fresh wt} - \text{Oven dry wt})}{\text{Fresh wt}} \times 100$$

The average MC of the five twigs becomes the predicted MC of the second 5-twig sample. A predicted oven-dry weight for each twig of the second 5-twig sample is calculated:

$$\text{Predicted Oven dry wt} = \text{Fresh wt} \times (1 - (\text{MC}/100))$$

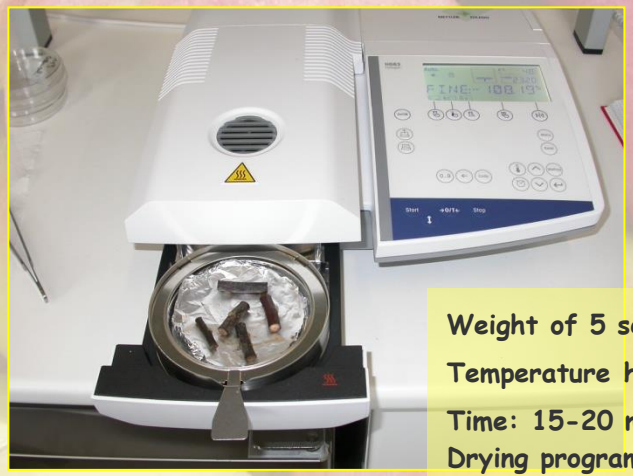
The second 5-twig sample is kept on the cafeteria tray with the rest of the twigs of that cultivar at –5°C. Individual twigs of the second sample are weighed every 2–3 days and their current MC is calculated as such

$$MC_{\text{day x}} = \frac{(\text{Fresh wt}_{\text{day x}} - \text{Predicted oven dw})}{\text{Fresh wt}_{\text{day x}}} \times 100$$

3. Moisture content determination → by Moisture Analyzer

⇩

Moisture content is determined every week (initially), then every 2-3 days by **Moisture Analyzer**

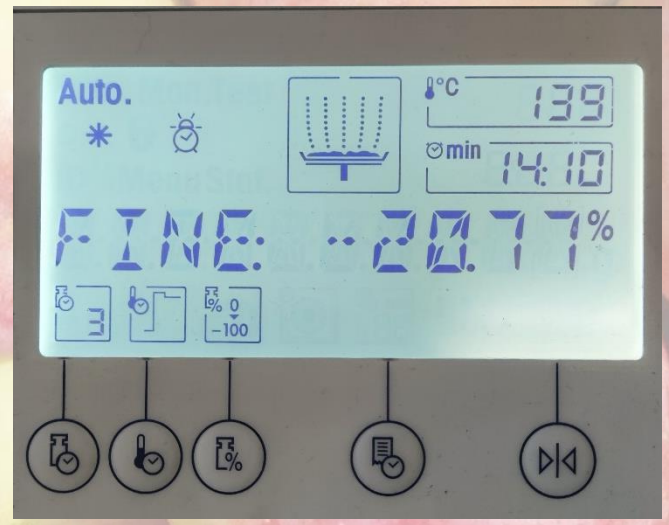


Weight of 5 segments ≈ 4-5 g

Temperature halogen lamp: 200°C

Time: 15-20 min

Drying programs: fast-drying



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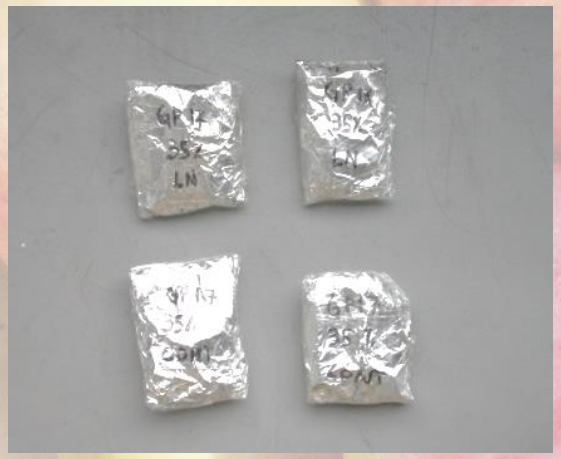
4a. Slow cooling, cryopreservation, thawing and rehydration

⇩

Desiccated micro-scions double wrapped in aluminium foil and plastic film to avoid futher desiccation and kept at – 5°C until used.



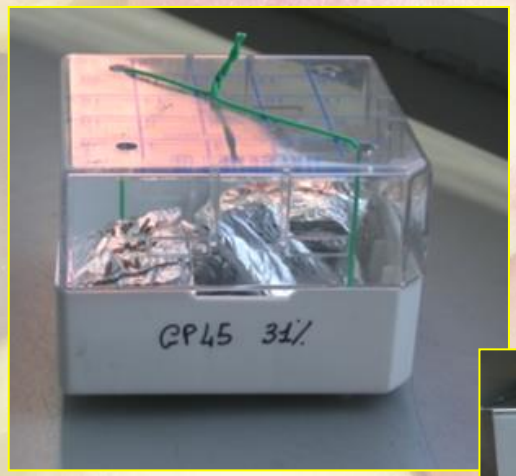
4b. Slow cooling, cryopreservation, thawing and rehydration

⇩

Micro-scions (still wrapped in alluminium foil) placed in cryobox and cooled in a controlled rate freezer at -1°C/h from –5°C to –30°C

⇩

Micro-scions kept at –30°C (24 h)



Sequence of temperature reduction →

08.00: - 6°C
09.00: - 7°C
10.00: - 8°C
11.00: - 9°C
12.00: - 10°C
13.00: - 11°C
14.00: - 12°C
15.00: - 13°C
16.00: - 14°C
17.00: - 15°C
18.00: - 16°C
19.00: - 17°C
20.00: - 18°C

OVERNIGHT

08.00: - 19°C
09.00: - 20°C
10.00: - 21°C
11.00: - 22°C
12.00: - 23°C
13.00: - 24°C
14.00: - 25°C
16.00: - 26°C
17.00: - 27°C
18.00: - 28°C
19.00: - 29°C
20.00: - 30°C

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4c. Slow cooling, cryopreservation, thawing and rehydration

⇩

Cryoboxes quickly removed from the blast chiller and immersed in LN

⇩

Cryobox rewarmed at 4°C (24 h)

⇩

Micro-scions rehydrated at 2°C in damp peat moss inside plastic containers (2 weeks)

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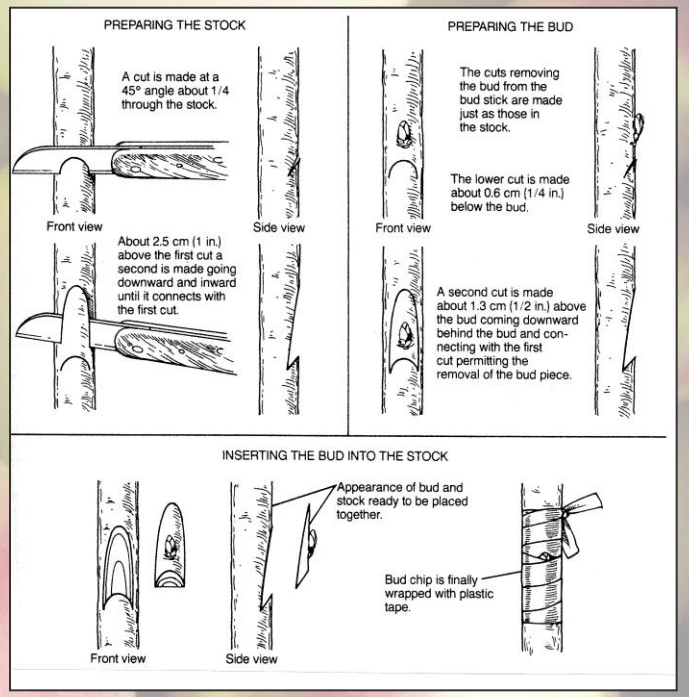
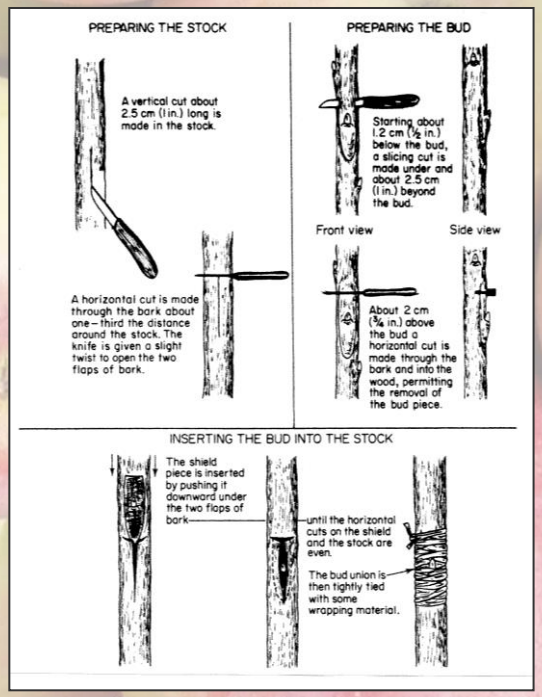
5. Grafting by chip-budding or T-budding

⇩

Rootstocks grafted with rehydrated buds (2 buds per roostock) by chip- of T-budding (late spring)



Chip budding



T-budding

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6. Post-budding operations and assessment of plant regrowth

⇩

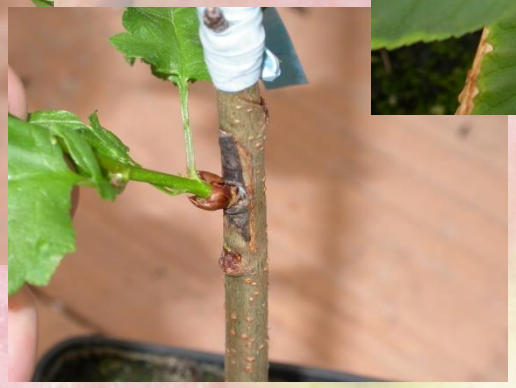
Grafted plants, kept in greenhouse, untied and cut about 50 mm above the grafted buds after 2-3 weeks

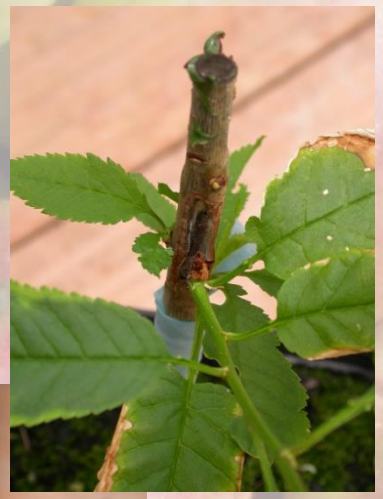
⇩

Grafted plants cut just above the top-grafted bud

⇩

Assessment of bud sprouting and plant regrowth (summer)





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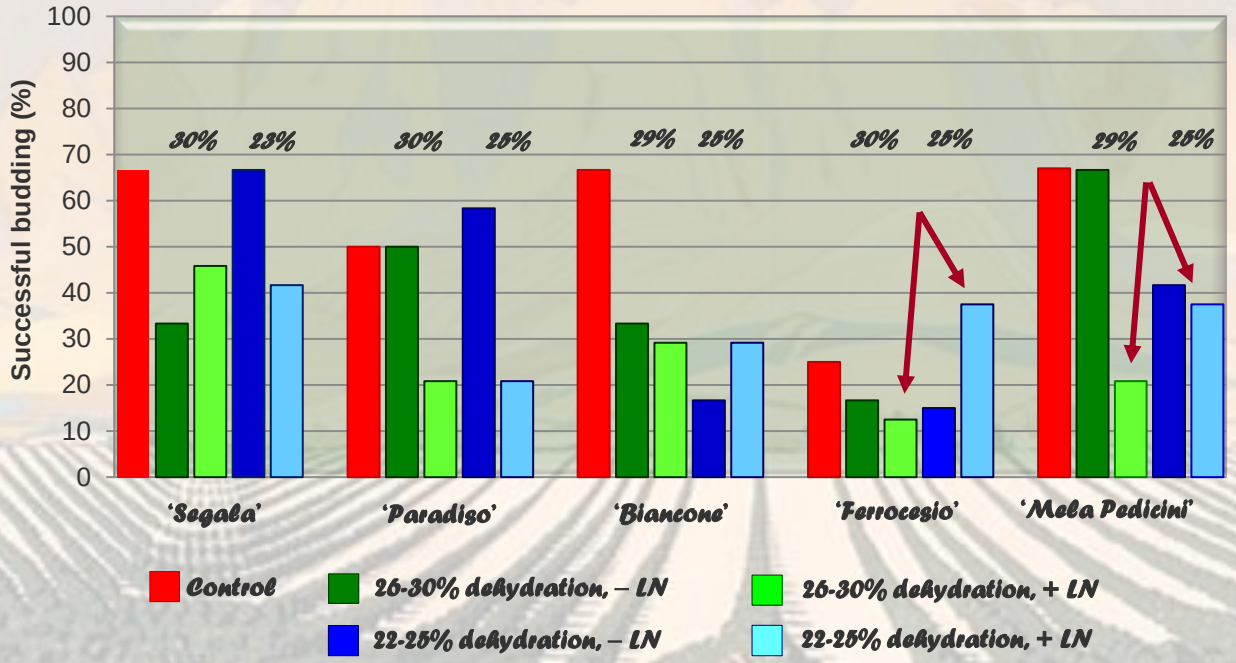
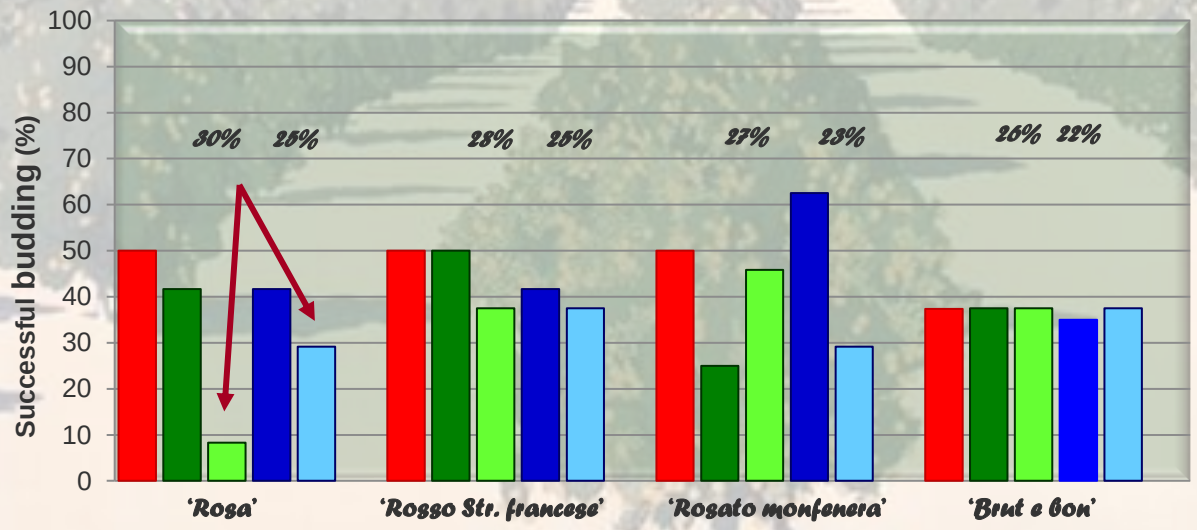
Some results → buds from trees in the field (year 2011)



Moisture content of micro-scions



Moisture content of micro-scions

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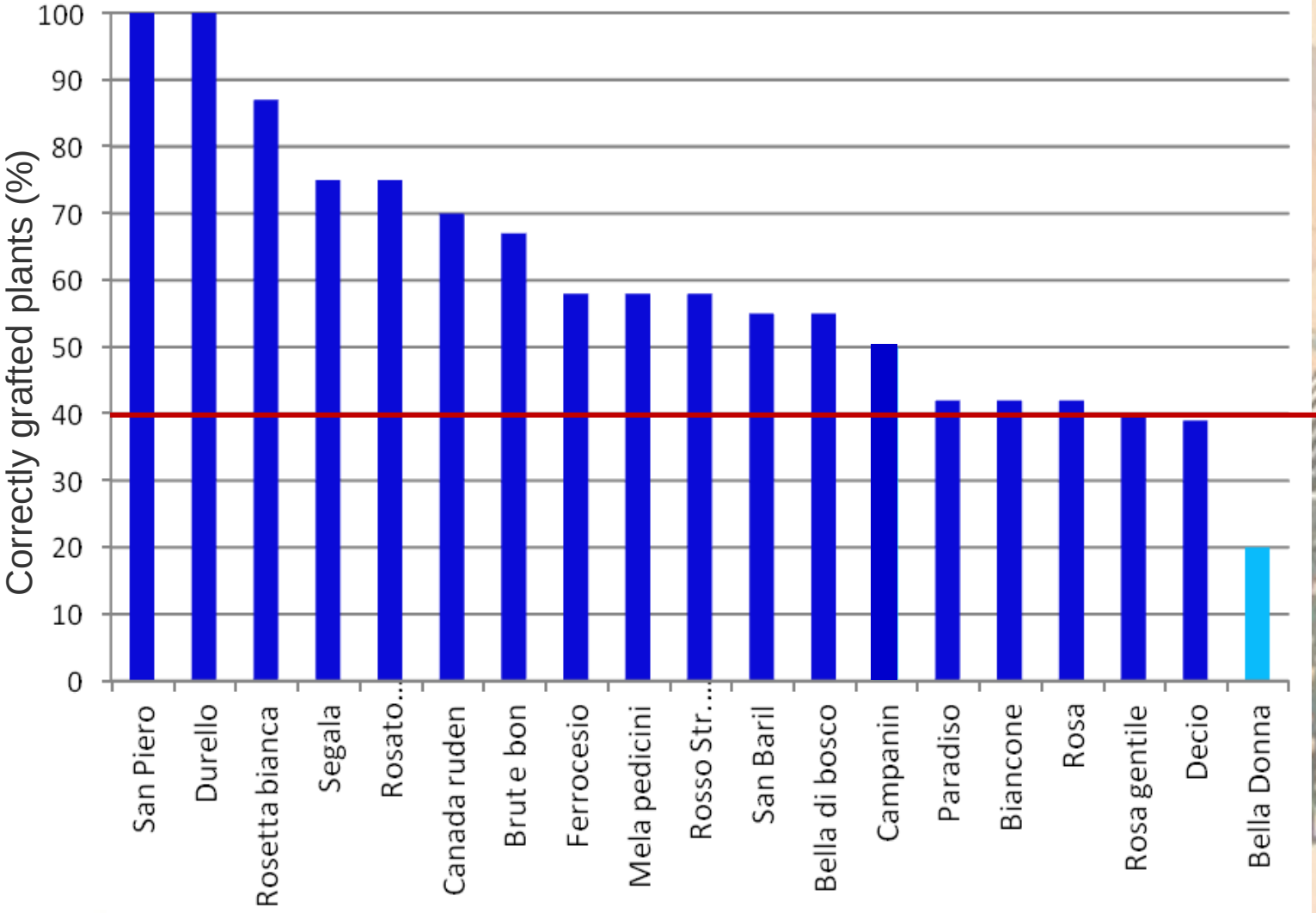
VENETO AGRICOLTURA

Some results → buds from trees in the field (years 2011-2015)



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Some results → buds from plants in screen-house

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-5°C, 2 mts

Micro-scion preparation

Dehydration to 22-25% MC

Moister analyzer

Chip budding

Re-hydration in moist peat, 2 wks

LN, -196°C

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Correctly grafted plants and regrowth

Variety	Clone	MC WDB	N° Rootstocks	N° Grafted WDB	% Regrowing plants (30 dd)	% Regrowing plants (60 dd)	% Regrowing plants (90 dd)
DEVIL GALA^{PVR}	ZACAV	26%	20	40	95	95	100
		23%	20	40	100	90	90
SIMMONSPVR BUCKEYE®	8150INCAV	26%	19	38	95	89	95
		23%	20	40	85	85	85
CAMSPUR RED CHIEF®	FNCAV	26%	20	39	90	85	90
		23%	20	39	80	80	50
GALAXY^{PVR}	CICAV	26%	30	60	93	83	83
		23%	30	60	90	77	77
GALAXY^{PVR} (da campo)	FNCCAV	26%	20	40	90	85	85
		23%	20	40	80	70	85
NORGE^{PVR} (da campo)	CICAV	26%	15	30	53	40	13
		23%	15	30	13	47	27

MC: moisture content; DB: winter dormant buds

Field test

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Camspur Red Chief

Cryopreservation of apple buds to assure a backup copy for pre-base materials held in screenhouses

M. Pancaldi[1], C. Benelli[2], F. Burroni[1], C. Contaldo[1], A. De Carlo[2], E. Tura[1] and M. Lambardi[2a]

Acta Horticulturae, in press

Mutant clones of polyclonal cultivars

Simmons Buckeye

Norge

Ongoing trials with other fruit species……..

Species	Accession	MC (%)	Nr. grafted rootstocks	Nr. grafted buds	Survival after LN (%)
Plum (*Prunus* spp.)	Blue free	24	10	20	**100**
	Sugar top	23	35	70	**68**
	President	22	12	24	**67**
	Blumoon	23	34	69	**52**
	Grossa di Felisio	23	35	70	**49**
	Fortune	24	14	28	**29**
	Zaipubo	20	18	36	**22**
	Agen	22	35	70	**20**
	Bianca di Milano	23	25	50	**12**
Pear (*Pyrus communis*)	Santa Maria	24	18	36	**22**
Cherry *Prunus avium*	Karima	23	19	38	**26**

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Problematics

The technique requires to be optimized for each cv, especially as for moisture content of uni-nodal sections before LN

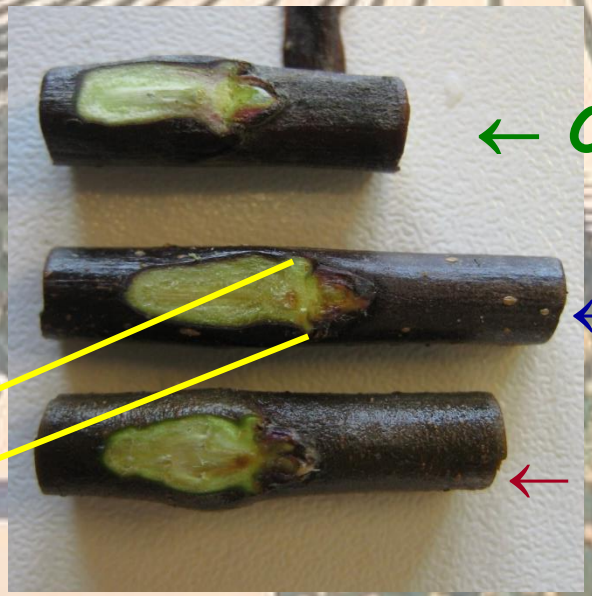
...but.....

- only one trial per year can be carried out, due to grafting time
- a large number of good-quality rootstocks is necessary every year

...hence.....

We consider fundamental to find precocious tests of viability for uni-nodal sections (buds+cambium cells), to be applied immediately after cryopreservation.

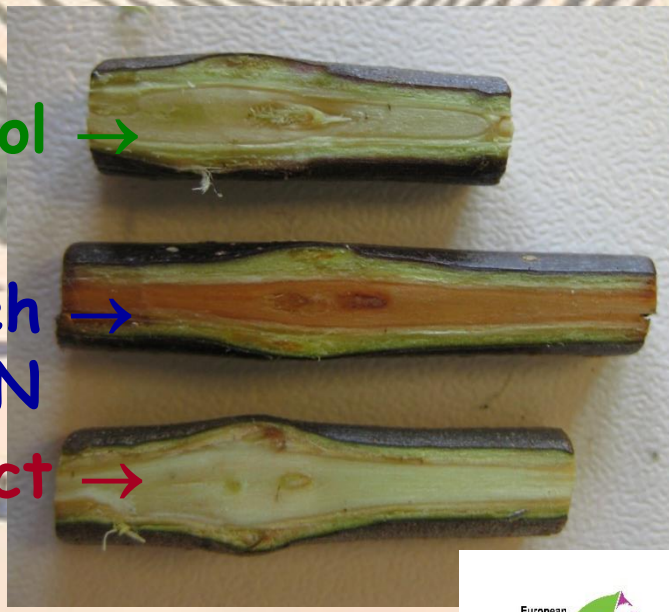
- A simple CUT TEST



← Control →

← Deh +LN →

← Direct LN →



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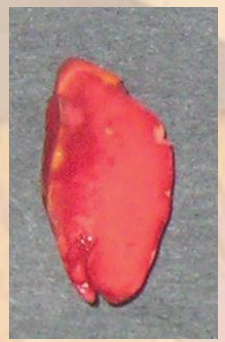
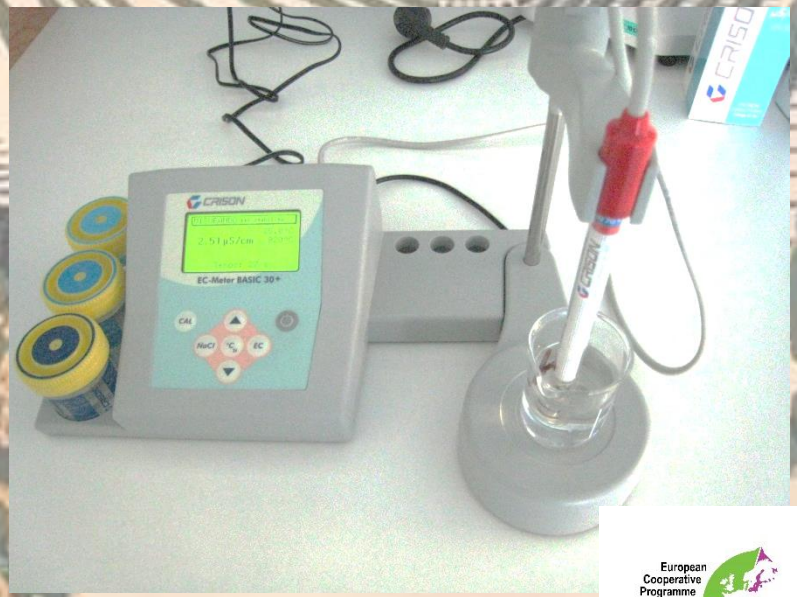
TTC (=TEZ) TEST (2,3,5-triphenyltetrazolium chloride)

→ commonly used to test seed viability. No information as for the use to test viability of bud and cambium cells.



ELECTROLYTE LEAKAGE TEST

→ conductivity determined by a conductance-meter Crison EC-BASIC 30, according to the method developed for oak seeds by Pasquini *et al.*, 2011



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TTC test

→ 0.5% TTC, 1 day at 30°C and darkness

Alive

Test applied immediately after cryopreservation and thawing

Dead

Electrolyte leakage → MEL test (Verleysen et al. 2004)

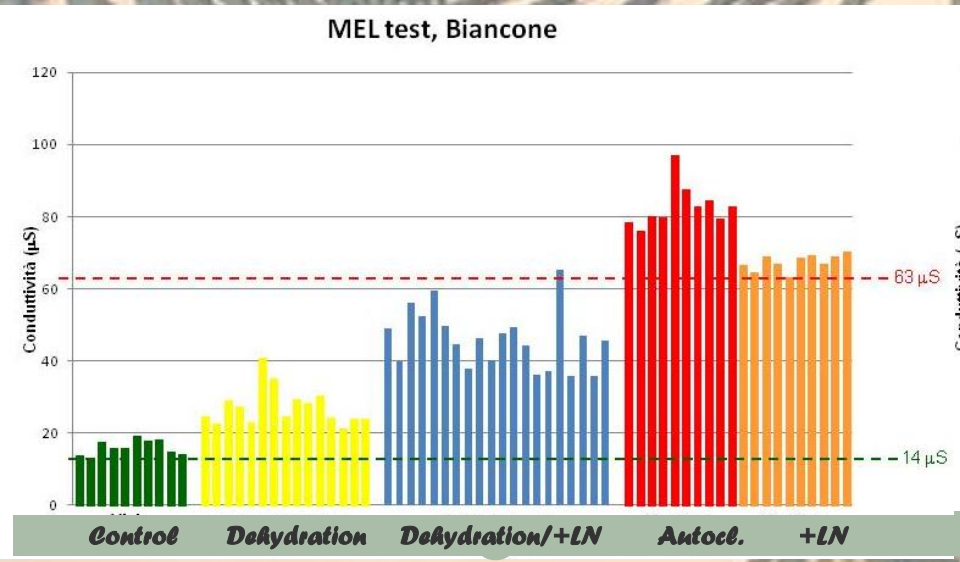
MEL test

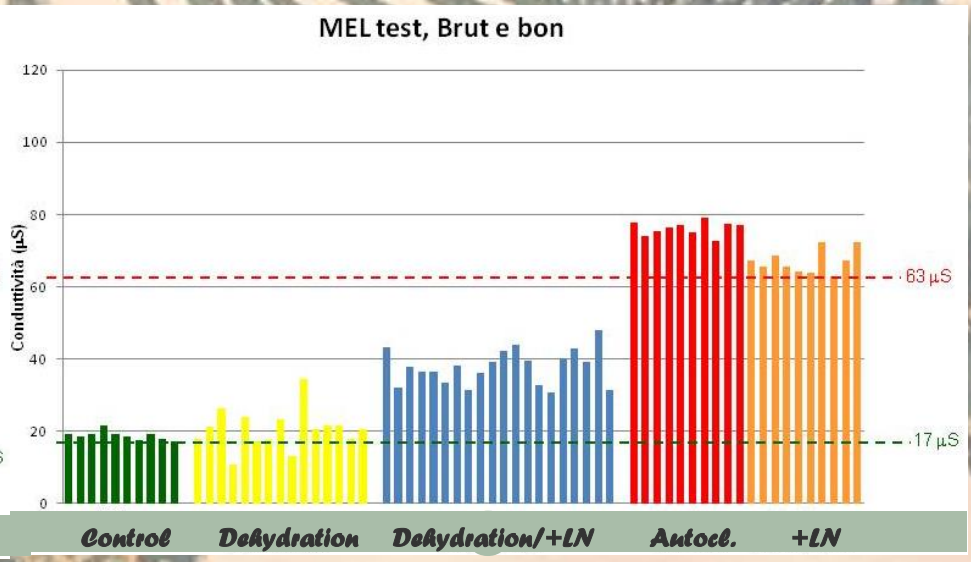
$C_{24} = 100 (C_x - C_0) / (C_K - C_0)$

where C_{24} is the conductance calculated after 24 hours of incubation, C_x is the measured conductance, C_0 is the conductance of the water and C_K is the value of the conductance measured after autoclaving the sample.

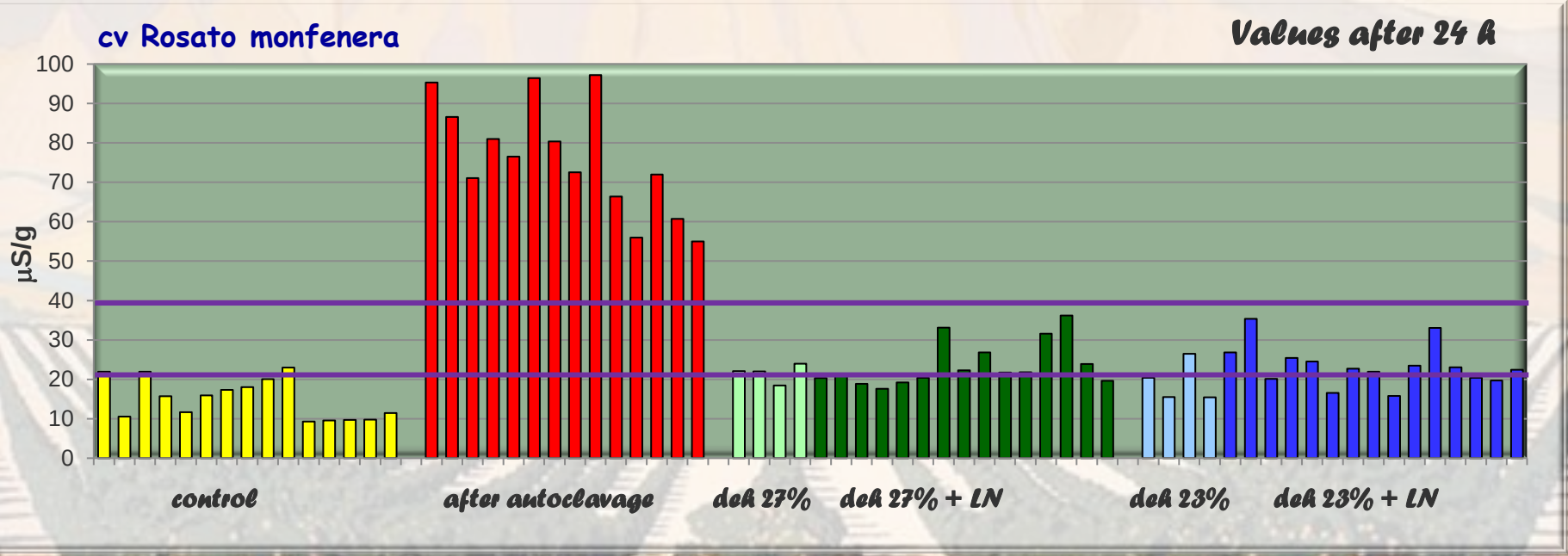
The test is based on the release of solutes in double-distilled water, following ruptures of the cell membranes. The greater the damage suffered by a plant tissue for any cause, the higher the quantity of solutes released from the cytoplasm and, consequently, the relative conductivity value of the water.

→ conductivity of bidistilled water measured after 8, 24 and 48 h and expressed in µSiemens/g of tissue

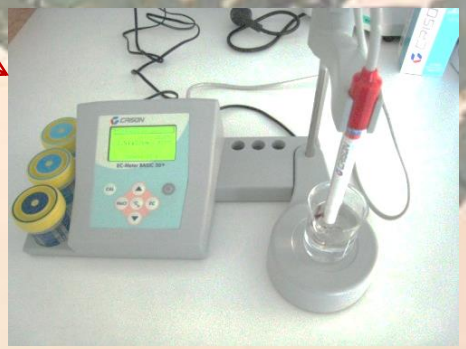




Electrolyte leakage test



Treatment	Successfull budding (%)	≤40 µS/g (%)
27% dehydration - LN	25	25
27% dehydration + LN	46	40
23% dehydration - LN	63	70
23% dehydration + LN	29	33



Optimizing a protocol for a single accession…….

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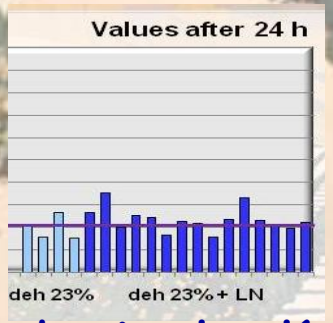
Winter scion collection

Dehydration-slow cooling treatments

Chip-budding

Preliminary tests of bud/cambium vitality



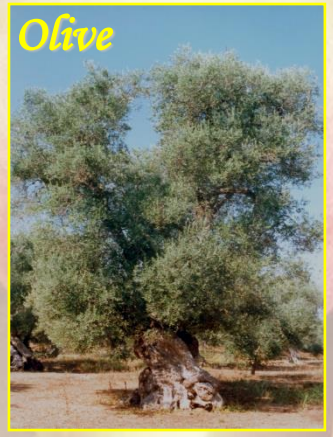
Presumed best dehydration level(s)

Problematics:

- the "species-specificity" of the technique

plum

apple



- *Not idoneous for species that cannot be reproduced by budding*

Olive grafting

CRYOPRESERVATION OF GRAPE (*Vitis vinifera*) DORMANT BUDS

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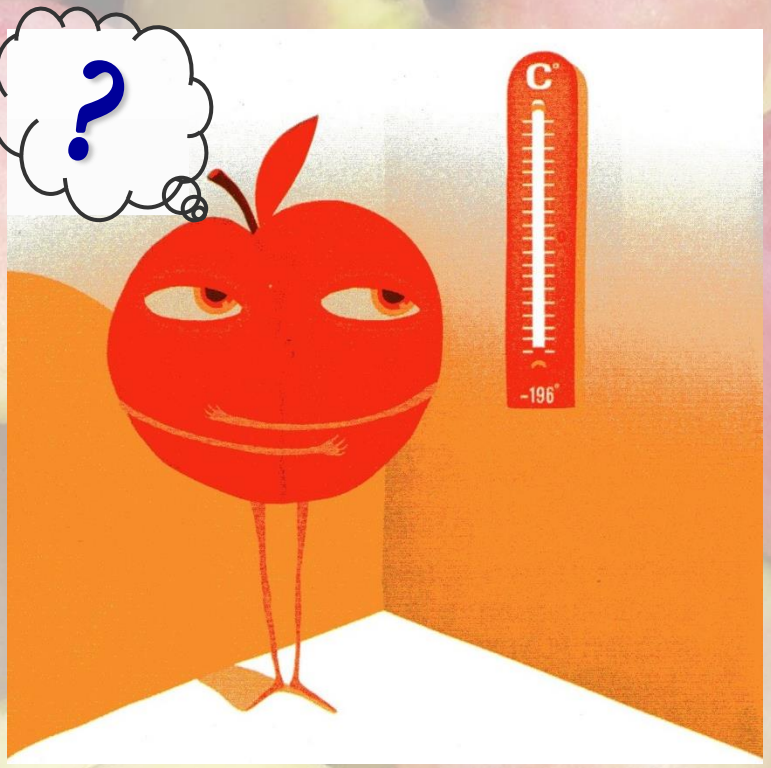


The question is now…..

…..which is the most efficient cryo-technique to duplicate an apple collection???

Lambardi M., Benelli C., De Carlo A., Ozudogru E.A., Previati A., Ellis D., 2012. Acta Hort. (1st Int. Symp. on Cryopreservation in Horticultural Species. Leuven, Belgio, 5-8 April 2009).

Apple cryopreservation by shoot-tip vitrification

Field ⇩ LN

JAN: scion collection and grafting in greenhouse

Mid FEB: introduction in vitro & establishment

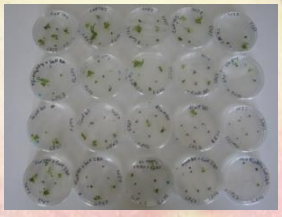
End MAY: shoot line for shoot tip excision

JUN: cryopreservation

Time per accession: 6 weeks → 3.5 months (4 subcultures) → 4 weeks = 6 mnt

Work per accession: 5 work hours → 6 work hours → 6 work hours = 17 wh

LN ⇩ Field



SEP: thawing, plating & shoot development

JAN: shoot line re-established

MAR: acclimatized plantlets

(MAY: plantation)

4 weeks → **16 wks (4 subcultures)** → **12 wks (elong+root+accl)** → ----- = 8 mnt

2 work hours → **8 work hours** → **4 work hours** → ----- = 14 wh

Apple cyopreservation by dormant-bud technique

Field ⇩ LN

Mid JAN: scion collection and cold-hardening

MAR: uni-nodal segment preparation & dessiccation

Mid APR: slow cooling & cryopreservation

Time per accession — 6 weeks + 6 weeks = 3 mnt

Work per accession — 1 work hour + 5 work hours = 6 wh

LN ⇩ Field

MAY: thawing and rehydration in damp peat

Mid MAY: budding

Mid MAY-End JUNE: graft healing, plant development, transfer to soil

2 weeks + 7 weeks = 9 wks

1 work hour + 2 work hours + 6 work hours = 9 wh

Dormant buds vs. PVS2 vitrification

Some advantages…..

- it doesn't require any passage *in vitro*
- from the field to LN in almost half time
- from LN to the field in 1/4 of the time
- about 50% of the handlabour required

Dormant buds vs. PVS2 vitrification

One disadvantage that cannot be underestimated…..

Dewar of 120 lt of liquid nitrogen

8 racks x 10 cryoboxes x
25 cryovials x 10 shoot tips
=
20,000 units of conservation

8 racks x 10 cryoboxes x
7 bags x 5 segments
=
2,800 units of conservation