Coconut Cryopreservation Training Course

IRD, Montpellier, France

13-17 October 2003

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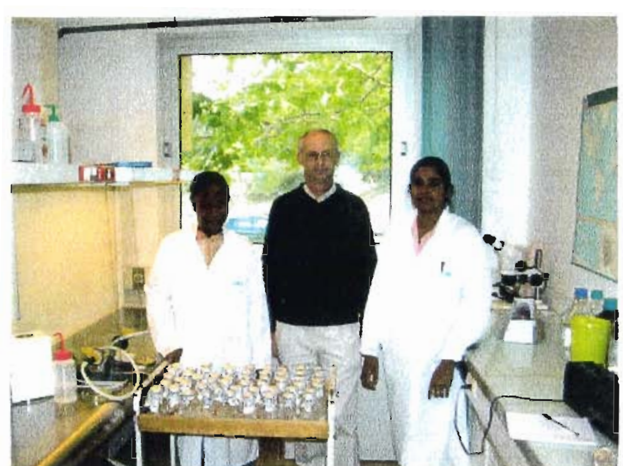
IPGRI-COGENT/IRD Coconut Cryopreservation Training Course, IRD, Montpellier, France
1st October 2003 – B. Malaurie



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Participants of the Coconut Cryopreservation Training Course, 13-17 October 2003, IRD Montpellier, France

Summary

<u>Complete Progamme</u>

Presentation Part,

Protocol Part:

Technical advice for plant conditioning and mailing

List of material needed

Abstract of the presentation part,

Prints and A4 Posters of the IRD/Cirad coconut team,

Addresses of the participants

Aknowledgement:

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The Coconut Cryopreservation Training Course was developed at IRD, Montpellier with IPGRI-Cogent initiative

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We thanks here all the Colleagues, from IRD and Cirad, for taking time in presentations and discussions with the four trainees coming from CICY (Mexico), EMBRAPA (Brazil), MARI (Tanzania) and PNGCCRI (Papua New Guinea). Thanks is also given to IRD in making possible this training course, allowing Mrs Prasanthi Perera (CRI, Sri Lanka), granted by IRD, to participate. Thanks is also given to Mrs Oulo N'Nan (PhD student from Côte d'Ivoire, granted by IRD) for her participation to the discussion with the trainees, to Colleagues working in the *in vitro* Culture Lab to have let the area free for one week. The final print of the report has been finalized with the help of Isabelle Hérault. Thanks are given to the Public relation and Communication service for the Photographs of the trainees team giving an overview during the Coconut cryopreservation training.

Time table of the Coconut Cryopreservation Training Course
IRD, Montpellier, France

Presentation part

13-17 October 2003

Monday 13th October:

Morning:

9h-9h15 (room 151)
1- Presentation of the program of the one-week training :
- Short welcoming,
- Practical aspects : money, work hours, restaurant, city travel,
- Details of the week programme.

9h15-9h45 (room 151)
2- **Official Welcoming at IRD :** in presence of JC Prot director/ De Noni IRD co-director, Yves Duval (Research Mixt Unit representative), André Rouzière (CIRAD-cp, Head of coconut research program),

9h45-10h: coffee break (room 151)

10h-11h45 (room 151)
3- **Short presentation by participants:**
- **10h-10h15** : Presentation of IRD (JC Prot director or/De Noni IRD co-director, and Valérie Rotival, Public relation and Communication Officer),
- **10h15-10h30** : Presentation of Research Mixt Unit (Yves Duval / Françoise Dosba),
- **10h30-10h45** : Presentation of coconut programme developed by Cirad (André Rouzière, CIRAD-cp, coconut
- **10h45-11h05; 11h05-11h15; 11h15-11h35; 11h35-11h55-**

Presentation by the welcomed trainees of their own Institutes and research programme,

11h55-12h30
4- **Visit of the IRD Laboratories** (Valérie Rotival)

12h30-14h
5- Lunch at IRD with all the participants: Room 151

Afternoom: -

1- Presentation of the coconut biotechnology programs developed at IRD by Valérie Hocher and Jean-Luc Verdeil.

14h-14h30: **Jean-Luc Verdeil** (Somatic embryogenesis), (room 151)
14h30-15h: **Valérie Hocher** (cellular cycle) (room 151)

15h-15h30 (room 151)
2- Presentation by Florent Engelmann of cryopreservation generalities,

15h30-16h (room 151)
3- Presentation of Oulo'works on coconut cryopreservation of complete zygotic embryo and plumule
Part one: the big lines of Oulo's work developed on coconut.

16h-17h30 (Tissue culture laboratory)

Tuesday 14th October:

Morning: (Tissue culture laboratory)

Afternoom: (Tissue culture laboratory)

Wednesday 15th October:

Morning: (Tissue culture laboratory)

Afternoom:

14h-14h45 (Room 151)
1- Presentation of Oulo'works on coconut cryopreservation of complete zygotic embryo and plumule. **Part two**: A more adapt presentation to answer point to point to the different question which could appear after these first days training.

2-. (Tissue culture laboratory)

3- (Tissue culture laboratory)

Thursday 16th October:

Morning: (Tissue culture laboratory)

Afternoom: (Room 108)

14h-15h
1- Presentation with Jean-Luc Verdeil of the different steps of histology, with the observation of the corresponding slides for the treatments used (sucrose/dehydration/cryopreservation).

15h15-15h45
2- Presentation by Jean-Luc Verdeil of the "Plant histo-cytology and cell imaging unit"

Friday:

Morning: (Room 151)

1- Meeting of people involved in cryopreservation and dehydration aspects at IRD,

9h15
- **Stéphane Dussert**: *The value of using DSC (Differential Scanning Calorimetry) analysis in seed cryopreservation studies.*

10h → Coffee break

10h15-11h
- **Frédérique Aberlenc**: *Physiological and molecular basis of the desiccation tolerance in palm oil zygotic and somatic embryos,*

2- Short presentations on oil palm biotechnologies:

11h-11h30
- **Yves Duval** (Biology of the development of cultivated perennial tropical plants group head, IRD)

11h30-12h
- **Alain Rival** (Head of Oil Palm Program, CIRAD)

12h-12h30
3- Meeting of CIRAD researchers involved on coconut (to be define with A. Rouzière).

Afternoom up to 15h30/16h30: (Room 151)

14h-14h30
1- Conservation strategies (Florent Engelmann),

14h30-15h00
2- Presentation of action plans by participants,

15h00-15h15
3- Evaluation of training course,

15h15-15h45
4- Problem solving, conclusion of the training

15h45-16h00
5 - Closing ceremony and delivery of training certificates.

Time table of the Coconut Cryopreservation Training Course
IRD, Montpellier, France

Protocol part

13-17 October 2003

Monday 13th October:

Morning: Welcoming presentation (Room 151)

Afternoon: -

1- Presentations 14h-16h (room 151)

2- Laboratory training 16h-17h30 (Tissue culture laboratory)

Albumen core containing zygotic embryos are disinfected after their extraction in the sending country in 100% commercial bleach, rinsed with sterile water and packed in sterile polystyrene container, refrigerated overnight before sending by plane (cf. in Annex: Coconut embryo obtaining and conditioning for mailing invoice).

Disinfecting of the albumen core brings by the participants and storage at 5°C.

The received albumen core containing zygotic embryos, are then one more disinfected:

- 12.5 % solution of sodium chlorate (250/2000ml) for **20 minutes,**
- Rinsed with sterile water 3 times,
- Distributed and stored in sterile container in a refrigerated obscured room.

Tuesday 14th October:

Morning: (Tissue culture laboratory)

1- Extraction of embryos (9h-9h30)

- Excised the zygotic embryo from the albumen core with a scalpel,
- Collect the embryos in a Petri dish

2- Disinfecting of the embryos (9h30-12h30)

- Do a final disinfecting before the plumule excision:
    - Collect the embryos and put them in a flask,
    - Add sodium hypochlorite (12.5 %), during **5 minutes,**
    - Rinsed with sterile water (use a sieve for long and easy water rinsing),
    - Collect the disinfected embryo in a Petri dish with some drops of water,
    - Divide in two plots of embryos:
        - 20 embryos,
        - 30 embryos

3- Dehydration step for complete zygotic embryo (9h30-9h45)

- Take the first batch of 20 embryos,
- Divide in four plots,
- Take the Petri dish containing medium,
- Put Five embryos over the medium,
- Put the filled Petri dishes over the silica gel,
- Note the starting hour of the dehydration.

9h45-10h; Coffee break

4- Plumules excision (10h-12h30)

- Take the second batch of 30 embryos,
- Excised the plumule under a stereo microscope,
- Transfer the excised plumule on Petri dish containing standard medium
    - 5 plumules by Petri dish.
- Place the Petri dishes in a dark room.

Afternoon 14th October: (Tissue culture laboratory)

5- Encapsulation of the plumules (14h-15h30)

Preparation of the little material used for this step:

a- unwrap the empty sterile flasks,
b- wash the pipette with alcohol,
c- take into the sterile flask the “sterile inert, bounded fiber barrier” and place it into the bottom edge of the 5000 µL appropriate pipette,
d- take into the sterile flask the 5000µl tip and place it on the 5000 µL pipette,
e- burn the blade until the red and cut the edge of the 5000 µL tip in a transversal way,

Encapsulation step:

a- Bring from the dark tissue culture room the 4 Petri dishes,
b- Take off the plumule from the Petri dish,
c- And place it over the surface of the sodium alginate solution,
d- Take the excised plumules by pipetting them,
e- Let the drop fall into the CaCl2 solution with a plumule in each drop,
f- Wait for **at least 20 minutes**, from the last beads, to insure a complete polymerization of the alginate beads

2- Sucrose pretreatment (15h30-16h30)

a- Surface-dry the CaCl2 remained over the beads, on sterile filter paper Diameter 7 cm,
b- Sort out the beads with apices from those without,
c- Take off carefully the polyurethane cap of the Erlenmeyer,
d- Burn the neck of the Erlenmeyer
e- Pick up the apices embedded in alginate beads and drop them into the sucrose solution (up to 20 embedded apices in each flask),
f- Replace the polyurethane cap and seal it with an extensible transparent film,
g- Mark on each Erlenmeyer the specific condition,
h- Place the Erlenmeyer on the rotary shaker,
i- Start on the shaker on 90 rpm.
j- Leave the Erlenmeyer on the rotary shaker up to the next days.

Wednesday 15th October:

Morning: (Tissue culture laboratory)

1- Dehydration of the plumule (9h-16h30)

For 6 hours dehydration duration:

a- Take off the Erlenmeyer from the rotary shaker,
b- Burn the neck of the Erlenmeyer
c- Take out the beads with apices from the Erlenmeyer with spatula,
d- Surface-dry the sucrose remained over the beads, on sterile filter paper Diameter 7 cm,
e- Weight the empty basket made with the sterile filter paper (7 cm diameter) (FPB),
f- Pick up the apices embedded in alginate beads and put down them over the (FPB) = (FPB+B)
g- Weight them on the Balance under laminar air flow cabinet (FWto),
h- Take the (FPB+B), open the airtight boxes and put it down to the silica gel,
i- Shut carefully the airtight boxes,
j- Note the hour, and mark the correspondent references (Molarity, desiccation duration, genotype), ***Start dehydration at 10 am***
k- Leave the airtight boxes under the laminar air flow cabinet until the end of the desiccation,
l- Weight them on the Balance under laminar air flow cabinet at the end of the desiccation (FWtx) → **STOP dehydration at 4 pm**,
m- For the dehydration control, transfer on culture medium half number of dehydrated-embedded plumules.

2- Stopping dehydration step for *complete zygotic embryo* and direct freezing into Liquid Nitrogen (11h-11h45)

a- Prepare sterile cryotubes or vials,
b- Take, at 11 pm, from an airtight boxes, **10** desiccated zygotic embryo and introduce them into sterile cryovial,
c- Do not forget to mark the vial with a pencil,
d- Put the vials on an aluminium cane and plunge them rapidly directly into liquid nitrogen,
e- Wait at least **1 hours** before taking them out from liquid nitrogen,
f- Transfer the other **10** desiccated zygotic embryo (control) on the standard culture medium.

3- Observation of the corresponding treatments:

-> plumules recovery after X months
-> Pictures / Study of results obtain

Wednesday Afternoon 15th October: (Room 151 & Tissue culture laboratory)

1- Presentation 14h-14h45 (Room 151)

2- Laboratory Training (Tissue culture laboratory)

A- Extraction from LN and Rapid rewarming (14h45-15h30)

Rapid rewarming

a- Take out the aluminium cane containing the vials from the canister,
b- Put it directly in the Water bath (30°C), for 3 min,
c- Transfer them on a standard culture medium.

Plant material:

Rapid rewarming of complete frozen dehydrated embryos → transfer on culture medium. (Tissue culture laboratory)

Rapid rewarming of freezed-6h dehydrated encapsulated plumules → transfer on culture medium.

B- Dehydration of the plumule (15h-16h30)

For 18 hours dehydration duration:

n- Take off the Erlenmeyer from the rotary shaker,
o- Burn the neck of the Erlenmeyer
p- Take out the beads with apices from the Erlenmeyer with spatula,
q- Surface-dry the sucrose remained over the beads, on sterile filter paper Diameter 7 cm,
r- Weight the empty basket made with the sterile filter paper (7 cm diameter) (FPB),
s- Pick up the apices embedded in alginate beads and put down them over the (FPB) = (FPB+B)
t- Weight them on the Balance under laminar air flow cabinet (FWto),
u- Take the (FPB+B), open the airtight boxes and put it down to the silica gel,
v- Shut carefully the airtight boxes,
w- Note the hour, and mark the correspondent references (Molarity, desiccation duration, genotype), ***Start dehydration at 4 pm***
x- Leave the airtight boxes under the laminar air flow cabinet until the end of the desiccation,
y- Weight them on the Balance under laminar air flow cabinet at the end of the desiccation (FWtx) → **STOP dehydration on Thursday at 10 am**,
z- For the dehydration control, transfer on culture medium the dehydrated-embedded plumules.

Thursday 16th October:

Morning: (Tissue culture laboratory)

1- Freezing/rapid rewarming and *in vitro* culturing.
at 10 am : Stopping of the **18h** dehydration duration

After 18 hours dehydration duration:

g- Prepare sterile cryotubes or vials,
h- Take, at **10 am**, from an airtight boxes, half of the dehydrated encapsulated plumules and introduce them into sterile cryovial,
i- Do not forget to mark the vial with a pencil,
j- Put the vials on an aluminium cane and plunge them rapidly directly into liquid nitrogen,
k- Wait at least **1 hours** before taking them out from liquid nitrogen,
l- Transfer the other half dehydrated encapsulated plumules (control) on the standard culture medium.

Rapid rewarming

d- Take out the aluminium cane containing the vials from the canister,
e- Put it directly in the Water bath (30°C), for 3 min,
f- Transfer the frozen dehydrated encapsulated plumules on a standard culture medium.

Thursday Afternoom 16th October: (Room 108)

1- Presentations 14h-14h45 (Room 108)

Friday 17th October:

Morning: (Room 151)

1- Presentation 9h15-12h30

Afternoom: (Room 151)

1- Concluding training 14h-16h

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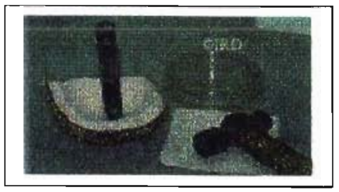
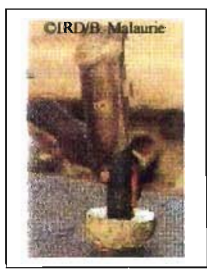
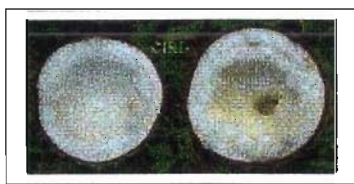
Coconut embryo obtaining and conditioning for mailing invoice

The first thing to do for the zygotic embryos obtaining is to excise and disinfect the albumen core which contain the embryo. For this step, we need a clean table, well washed and disinfected with commercial bleach solution.

Coconut seed needs to be completely mature (11 to 12 months). After parch removal from the nut, they need to be part in two with a machete or any strong metal bar. The cylindrical albumen core which contain the embryo could be obtain with the use of a punch, 3cm in external diameter, and forceps. All these material have to be previously disinfected by their immersion in a flask filled with a commercial bleach solution (12.5% chlorine). Before this, it is necessary to sterilise all the instruments over the flame of a gas burner.







Embryo location in the nut could be situated by taking germinate pores as reference level. The embryo inlayed into albumen is located under one of the three germinate pores which form the three eyes of the nut. The eye corresponding to the embryo could be determined by simple pressure (this small deformation is due to the very leak lignification that occur and is responsible to the exit of the embryogenic axis after that germination occur). But it is easier to fetch the embryo location when observed from the interior albumen side: in the bottom it is possible to see a hole which got a colour different from the surrounding tissue (lightly dark).

The cylindrical albumen cores which contain embryos are washed under clean tap water, then immersed during 5 min into 80% ethyl alcohol to cleaning off their external lipids excess. Their disinfecting is done by a 20mn immersion into a commercial bleach solution (12.5% active chlorine), followed by three washes with bidistillated sterile water. The rinsed cylindrical albumen cores containing embryos are then transferred to clean disinfected plastic bags. They are conditioned as follow 10 by 10 in small plastic bag, which are put together in a bigger plastic bag, up to 100, sealed before transportation in a polystyrene isothermal box filled with plastic bags containing freeze water. To assure a good transportation it is recommended to keep as long as possible the material under refrigeration before leaving it to the mailing post service (preferably DHL).

Type of conditioning in polystyrene isothermal box with albumen cores in plastic bag

List of Material needed for rapid and direct application of coconut cryopreservation process after Montpellier Cryo-Training, when using plumular tissues

Material and product needed for cryopreservation process

Different types of flasks used in a tissue culture laboratory :

- Airtight glass boxes (125 ml : 9 cm diameter; 4 cm high)

- Bechers,
- Erlenmeyer for sucrose solution (100 ml)

- Flasks of 100 ml for sodium alginate

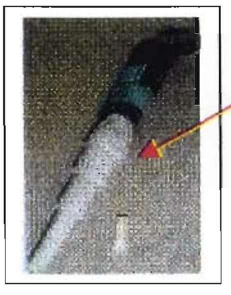
- Flasks of 250 ml for CaCl2

- Flasks of different volumes with caps,
- Graduates test tubes
- Sterile Petri dishes (90 mm diameter),
- Test tubes, or sterile Petri dishes (50mm diameter), filled with Eeuwens medium,

Different little material needed

- Forceps,
- Polyurethane cap, or sterile cotton caps,
- Parafilm or any extensible film used for food protection.
- Scalpels,
- Scalpels, with new blades,
- Spatula
- Sterile filter paper Diameter 7 cm, for the surface-dried of the CaCl2 remained over the beads,
- Sterile filter paper for the surface-dried of the sucrose remained over the beads,
- Sterile filter paper (diameter 5 cm) for the desiccation over silica gel,
- Sterile filter paper (diameter 7 cm), shaped as basket and sterilised in a glass jar,
- Micro-Pipette 5000 µL [Gilson; or Sigma...]
- "Sterile inert, bounded fiber barrier" **for corresponding pipettes *[1]**, to protect the pipette cavity from air-borne contaminant
- Pipette tips 5000 µL

[1] *** Careful !** Each mark of pipette have their own "sterile inert, bonded fiber barrier" size



Sterile inert, bounded fiber barrier, as a protection of the pipette cavity from air-borne contaminant

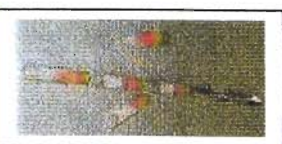
REMARKS:
For laboratories with low funding, it is possible to create alginate beads with more simple and cheaper material.

Different bigger instruments needed

- Balance under laminar air flow cabinet
- Oven up to 150°C overnight for airtight boxes sterilisation and silica gel regeneration,
- Oven, for the dry sterilisation of the scalpels, forceps
- Rotary shaker (90 rpm),
- Stereo Binocular,
- Water bath (30°C),

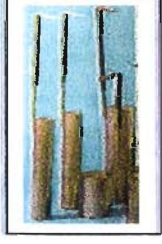
Different material specific to cryopreservation process

- Aluminium cane 1.2 cm diameter x 29 cm length, with tube stops, holds 5 tubes of 5 cm in height (example: Sigma C 3171)



Aluminium cane with 2ml cryovials

- Canister for storage and easy retrieval of vials in canes (example: Sigma C. 3421)



- Dewar container for short-term storage of liquid nitrogen (example: Sigma F. 9401 for 1 L

- Sterile cryotubes or cryovials (2 ml) , 5 cm in high (Corning, Nunc,...)

- Tank or container for ultra-cold storage for long-term storage (example: Sigma F 2344 for 35 L)

Tissue culture facilities

- Apparatus for bidistillated water,
- Apparatus for pH,
- Laminar air-flow cabinet.
- Tissue culture room. *Note that our experiments were developed in the following environmental conditions: temperature (27 ± 1°C) with obscurity*,

Products and solutions needed for cryopreservation process

- Liquid nitrogen
- Silica gel (Sigma S.7625, or Labosi, Ref. A 481 38 03),
- Sodium hypochlorite solution,
- Solution of CaCl2 100 mM (Labosi, Ref. A 475 93 51)
- Solution of sodium alginate (Sigma, 250 cps, A 2158)
- Sterile water,

Miscellaneous

- Freezer.
- Refregirator,

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Composition of the Tissue Culture Media Needed for the Cryopreservation of Coconut Plumules by the Encapsulation / Dehydration Process

Standard culture medium

❖ *Preparation*

Standard culture medium	V = 1L
Macro EEuwens solution	50 ml
Oligo EEuwens solution	10 ml
4 vitamins Morel & Wetmore (x1000)	1 ml
Biotine	2 ml
Ascorbic acid	100 mg
FeEDTA (Jacobson)	1 ml
Myo-Inositol	100 mg
Sodium phosphate	25 ml
Adenine sulfate	30 mg
Sucrose	40 g
pH	5
Gelrite (Phytagel)	4 g
Activated charcoal	2 g

After pH control, Gelrite and charcoal are added without heating, but under rapid shaking

Note that, when activated charcoal is added, sediment at the bottom of the the test tubes could be avoided by its homogenization and rapid gel solidification in a sink full of crushed ice

Encapsulation media *

Eeuwens mother solution WITHOUT $CaCl_2$

Macro Eeuwens	V = 1 L
KNO_3	40.40 g
KCl	29.84 g
NH_4Cl	10.70 g
$MgSO_4, 7H_2O$	4.94 g
Take 50 ml/l of the solution	
$NaH_2PO_4, 2H_2O$	12.48 g
Take 25 ml/l of the last solution	

❖ *Preparation of the common part solution used for alginate solution and for the CaCl2 solution*

Base solution	V = 2L in one Liter
Macro Eeuwens solution WITHOUT Ca Cl2	100ml
Oligo Eeuwens solution	20 ml
4 vitamins Morel & Wetmore (x1000)	2 ml
Biotine	4 ml
Ascorbic acid	200 mg
Sodium phosphate	50 ml
Adenine sulfate	60 mg
FeEDTA (Jacobson)	2 ml
Myo-Inositol	200 mg

Adjust the solution to 1L

500 ml → Sodium Alginate solution

500 ml → CaCl2 solution

Sodium Alginate solution	
Sucrose	50 g
Adjust to 1L	
pH	5
Heat the solution	
Sodium Alginate (3%)	30 g
Autoclave the solution	

CaCl2 solution	
Sucrose	50 g
$CaCl_2, 2H_2O$ (14.7 g/l)	14.7 g
Adjust to 1L	
pH	5
Autoclave the solution	

Mother solutions

Preparation of a mother solution for the Standard culture medium

Macro Eeuwens	V = 1 L	Oligo Eeuwens	V = 1 L	4 Morel vitamins	V = 500 ml
KNO_3	40.40 g	$Mn SO_4, H_2O$	848 mg		
KCl	29.84 g	KI	83 mg	**Ca Pantothenate**	500 mg
$NH_4 Cl$	10.70 g	$Zn SO_4, 7 H_2O$	72 mg	**Nicotinic acid**	500 mg
$Ca Cl_2, 2H_2O$	5.88 g	H_3BO_3	31 mg	**Pyridoxin HCL**	500 mg
$Mg SO_4, 7 H_2O$	4.94 g	$Co Cl_2, 6H_2O$	0.024 mg [1]	**Thiamin HCL**	500 mg
		$Na_2 Mo O_4, 2H_2O$	0.024 mg [2]		
Take 50 ml/l of the solution		$Cu SO_4, 5 H_2O$	0.016 mg [3]	**Take 1 ml of the solution**	
		$Ni Cl_2, 6 H_2O$	0.0024 mg [4]		
$NaH_2 PO_4, 2H_2O$	12.48 g				
Take 25 ml/l of the last solution					

1 Take a sample of 4.8 ml of a mother solution with 5 g/l
2 Take a sample of 4.8 ml of a mother solution with 5 g/l
3 Take a sample of 3.2 ml of a mother solution with 5 g/l
4 Take a sample of 0.48 ml of a mother solution with 5 g/l

Preparation of a FeEDTA solution (Jacobson modified):

* $Fe SO_4$, 7 H_2O and Na_2 EDTA, 2 H_2O are prepared alone in FeEDTA solution, such as below

1) Weigh 2.61 g of EDTA
2) Add 20 ml of KOH normal
3) Shake the solution
4) Add some more KOH normal until the pH reach 10.2 +- 0.1
5) Dissolve 2.49 g of $FeSO_4$, 7 H_2O in 30 ml distillate water
6) Add this solution to the solution of EDTA
7) Shake the mixture
8) Complete up to 100 ml with distillate water
9) Organize an air flow system which could the airing of the solution during one night (the pH change during the night until reaching 6.4 to 6.8)

→ → → 1 ml of this solution brig 5 ppm of iron.

CRYOPRESERVATION OF COCONUT (*COCOS NUCIFERA* L.) PLUMULES BY ENCAPSULATION/ DEHYDRATION.

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INTRODUCTION: First works on cryopreservation of complete zygotic embryos have been reported in 1992 (1). Meanwhile, complementary studies have been requested, through Cogent, by countries working on coconut. Previous histological studies have shown that only meristematic tissues survived to freezing. In this work we try to use for cryopreservation caulinary apices (plumules) excised from zygotic embryo where recent studies with photonic and electronic microscopy showed the different damages occurred in the cells and their degree of importance for their further survival (2, 3).

MATERIAL & METHODS: Plant material was MYD disinfected endosperm bore enclosing embryo coming from CICY, Yucatan. Plumules 0.5-1mm long, excised under stereo microscope, were encapsulated in alginate beads. Encapsulation, sucrose pretreatment, dehydration process were those described on (4) adapted to coconut conditions. Pretreatment was done over 0.5, 0.75 and 1M sucrose and desiccation over 8, 10, 16 and 24h. Medium used along the process and culture was Eeuwens (5) modified. Results were done according to observations at 1 and 6 months of survival.

RESULTS & DISCUSSION: At the first month of culture the best result was obtained for 1M sucrose and 10h desiccation, with 40% survival. At 6 months culture, better results were obtained with the two sucrose concentrations, 0.75 and 1M, from 8 to 16h dehydration. Other experiments are underway to confirm the good range of treatments over a large number of genotypes.

Table 1: Effect of different treatments in survival rate of encapsulated plumules at 6 months (%).

Sucrose concentrations (M)		***Dehydration duration (h)***			
		8	***10***	***16***	***24***
0.5M	+AL	16.7			20
	-AL	11		10	
0.75M	+AL	66.6	60		
	-AL	33	30	33	
1.0M	+AL	46.5		50	22
	-AL		10	57	30

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1. Assy-Bah & Engelmann. Cryo-Letters, 13: 117-126; 1992.
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4. Malaurie *et al.* Cryo-Letters, 19: 15-26; 1998.
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Acknowledgements: This work was carried out in Montpellier in IRD laboratory, with the IRD/CIRAD coconut team, following a two months IRD ESCD grant.

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Coconut (*Cocos nucifera* L.) cryopreservation of caulinary meristems : research of an optimal process with encapsualtion/dehydration technique.

Photos: Bernard Malaurie

Oulo N'nan [1], Misterbino Borges [2], Bernard Malaurie [1]

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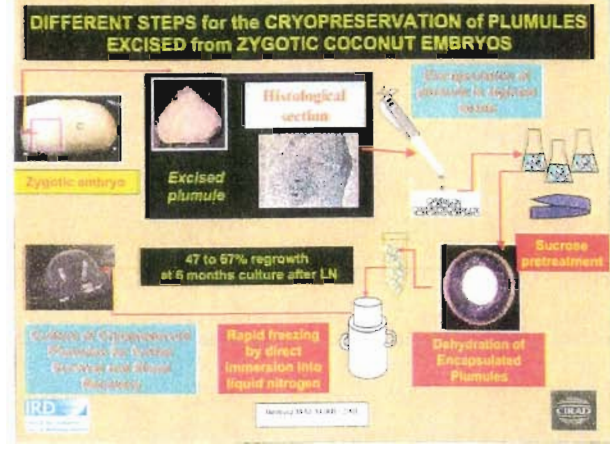
Introduction: If first works on cryopreservation of complete zygotic embryos have been reported in 1992 (1), focus on conservation of coconut genetic resources need still to be explored, because of increasing genetic erosion. After previous histological studies, showing that only meristematic tissue survived to freezing step, works were focus on caulinary apices (plumules) excised from zygotic embryo. In these works recent studies with photonic and electronic microscopy showed the different damages occurred in the cells and their degree of importance for their further survival (2, 3); whereas, 47 to 67% incomplete regrowth was observed with MYD Mexican plumules (4).



Figures: 1) Coconuts; 2) Open coconut with hole of the missing albumen core containing embryo; 3) Type of packaging for shipment; 4) embryo excision; 5) MYD embryos; 6-7) plumule excision.

Marerial & Methods:

Material: Plumular tissues excised from zygotic embryos of MYD coconut (*Cocos nucifera* L.) from CNRA, Côte d'Ivoire.

Technique: encapsulation/dehydration

Sucrose pretreatment: 0.75 to 1.1M; ***Dehydration :*** 8 to 24h; ***Freezing:*** rapid immersion into liquid nitrogen (-196°C); ***Rapid thawing***

Figures: 1) plumules embedded in alginate beads; 2) Weight of beads under aseptic conditions; 3) dehydrated beads; 4) direct immersion into liquid nitrogen; 5) First swelling; 6-7) type of plumule regrowth after 4 months of culture.

Results :

Controls:

- 100% growth potential with:
  - 25% swelling and 75% shooting

At 3rd month:

- sucrose 0.9M → 16% growth potential
- 10h dehydration → 32% growth potential

At 6th month:

- between 10 to 20% shooting depending treatments

References:

1. Assy-Bah & Engelmann. Cryo-Letters, 13: 117-126; 1992.
2. N'Nan, IRD/CIRAD coconut team, ESCD training report, 1999.
3. Malaurie *et al.* Proc. " 2nd Intern. Coco. Emb. Cult. Workshop ", Cogent-IPGRI, CICY, Mérida, Mexico, 14-17 March 2000, (in press).
4. Malaurie & Borges, BIOVEG 2001, Intern. Workshop – Centro de Bioplantas, Ciego de Avila, Cuba. Short Report Abstract p59., 2001.

Acknowledgements : This work was carried out with the **IRD/CIRAD** coconut team, in the **IRD Centre of Montpellier** with a three years **IRD BSF** grant and one year **IRD ESCD** grant, respectively; and it was done feasible with support of regular material invoice provided by Marc Delorme Research Station (**CNRA**, Côte d'Ivoire).

Research of an optimal cryopreservation process using encapsulation/ osmoprotection/ dehydration and encapsulation/osmoprotection /vitrification techniques on caulinary meristems of coconut (*Cocos nucifera* L.).

Bernard Malaurie [1], **Misterbino Borges** [2], **Oulo N'nan** [1]

Photos: Bernard Malaurie

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Introduction: First works on coconut cryopreservation have been reported in 1992 (1), using complete zygotic embryos. Considering that only meristematic tissues survived to freezing (2), complementary studies were developed using caulinary apices (plumules) excised from zygotic embryos (3). In this work we try to investigate other techniques than encapsulation/dehydration (4) on plumules cryopreservation using in addition osmoprotection, vitrification according to those developed by Sakai *et al* (5).

Marerial & Methods:

Material: Plumular tissues excised from zygotic embryos of MYD coconut (*Cocos nucifera* L.)

Techniques: (I) encapsulation/osmoprotection/dehydration and (II) encapsulation/osmoprotection/vitrification

Dehydration : (I) 2 to 10h; (II) 20 to 100min; ***Freezing:*** rapid immersion into LN -liquid nitrogen- (-196°C); ***Rapid thawing***

Results :

- *Controls:* 100% growth potential
- *(I):*
  - 100% swelling for most of the treatments
  - Best results allowed to obtain up to 25% shooting
- *(II):*
  - 100% swelling for -LN treatments
  - Best results allowed to obtain shooting up to 11% with shorter dehydration duration

Figures: 1) preculture ; 2) immersion into a solution added with osmoprotectors; 3) dehydration with silicagel; 4) rapid immersion into liquid nitrogen; 5) rapid thawing; 6) In case of technique (II) elimination of the PVS2 solution; 7) subculture of the embedded plumules on standard semi-liquid medium for short term ; 8) development of the plumule after 3 months (-LN); 9) development of the plumule after 1 month (+LN).

References:

1. Assy-Bah & Engelmann. Cryo-Letters, 13: 117-126; 1992.
2. N'Nan, IRD/CIRAD coconut team, ESCD training report, 1999.
3. Malaurie *et al.* Proc. " 2nd Intern. Coco. Emb. Cult. Workshop ", Cogent-IPGRI, CICY, Mérida, Mexico, 14-17 March 2000.
4. Malaurie *et al.* Cryo-Letters, 19: 15-26; 1998.
5. Sakai et al. Cryo-Letters, 21: 53-62; 2000.

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Are caulinary meristems suitable for cryopreservation of coconut (*Cocos nucifera* L.) with encapsualtion/dehydration technique ? Histological studies, a way to find the good range of pretreatments.

IRD Institut de recherche pour le développement

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Photos: ©IRD / Bernard Malaurie

Introduction: Cryopreservation of plumular tissues dissected from coconut zygotic embryos needs to be able to have very early information of reactions to stress of the cells and their hypothetical degree of damages .

Results obtains from optic and electronic histological analysis after dehydration and freezing appeared to give us a good idea of the cell behaviour in regard to the different condition stress.

Results:

In vitro culture

Survival rate

No dehydration: 80%
Dehydration without freezing: 20 to 50%
Dehydration and freezing: 20 to 50%

Recovery rate

No dehydration: 80%
Dehydration without freezing: 0 to 30%
Dehydration and freezing: 0 to 20%

No effect of freezing, but effect of dehydration

Material & Methods:

Material:

Ecotye: Malayan Yellow Dwarf (MYD); Matures seeds; Plumulars tissues: apical dome with two to four leaf primordia; Origin: CNRA, Côte d'Ivoire.

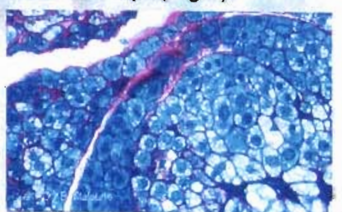
Methods:

Encapsulation-dehydration technique: 1) Pretreatment with sucrose (0.75M to 1M); 2) Dehydration with silica gel (8h to 24h); 3) Rapid freezing; Rapid thawing at 40°C during 3mn; 4) Subculture on Eeuwens medium.

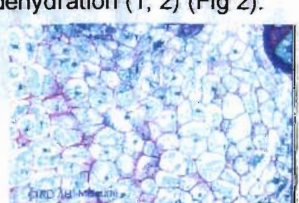
Histological studies

After dehydration:

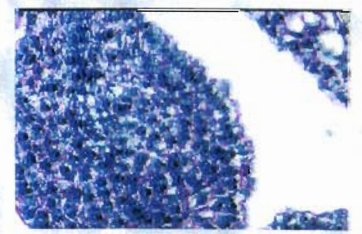
During short time (4h to 6h). No plasmolysis of the cells from the apical dome and the young leaf primordia. Cells from the other part are slightly plasmolysed, but no change of nucleus shape(Fig 1)



During intermediate time (8h to 16h). The cells of the apical dome and the young leaves are slightly plasmolysed with morphological change of nucleus, chromatin condensation. The same observation have been observed in sugar cane and coffee and shoot tips after dehydration (1, 2) (Fig 2).



During long time: (18h to 24h). Most of cells are destroyed. Some cells appeared without nucleus. (Fig 3)



After freezing

We notice the same observation. The only difference was when dehydration was performed during short time. In this case, all the cells are destroyed after freezing. It was impossible to distinguish cells individually. The walls and cytoplasm membrane are damaged (Fig 4).

Conclusion: The use of histological studies to choice the best treatment give us a great number of indication of the degree of stress and damage of the cell after cryopreservation /dehydration treatments. Meanwhile, as material used is composed by seeds in free pollination, where seeds could be different from each other, it is not certain to have the same case in an other time. Nevertheless, it make us understand some effect of dehydration and freezing.

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" BioVeg 2003 ", 14-19 April 2003, Ciego de Avila, Cuba

Nominations for IPGRI-COGENT funded participants to Coconut Cryopreservation Training Course at Institut de recherche pour le développement (IRD), Montpellier, France
13 - 17 October 2003

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Coconut Cryopreservation Training Course

IRD, Montpellier, France

13-17 October 2003

PRESENTATIONS
Abstracts

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IRD Cryoconservation du cocotier

Summary

Presentation

Abstracts of the presentation

Addresses of the participants

Aknowledgement:

This training course is funded in part by the Common Fund for Commodities (CFC) - funded project of IPGRI entitled "Coconut Germplasm Utilization and Conservation to Promote Sustainable Coconut Production". The CFC is an intergovernmental organization, headquartered in Amsterdam, the Netherlands.

The Coconut Cryopreservation Training Course was developed at IRD, Montpellier with IPGRI-Cogent initiative

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SERDANG, 43400, Malaysia

We thanks here all the Colleagues, from IRD and Cirad, for taking time in presentations and discussions with the four trainees coming from CICY (Mexico), EMBRAPA (Brazil), MARI (Tanzania) and PNGCCRI (Papua New Guinea). Thanks is also given to IRD in making possible this training course, to Colleagues working in the *in vitro* Culture Lab to have let the area free for one week. The final print of the report has been finalized with the help of Isabelle Hérault. Thanks are given to the Public relation and Communication service.

Time table of the Coconut Cryopreservation Training Course
IRD, Montpellier, France

Presentation part

13-17 October 2003

Monday 13th October:

Morning:

9h-9h15 (room 151)
1- **Presentation of the program of the one-week training :**
- Short welcoming,
- Practical aspects : money, work hours, restaurant, city travel,
- Details of the week programme.

9h15-9h45 (room 151)
2- **Official Welcoming at IRD :** in presence of JC Prot director/ De Noni IRD co-director, Yves Duval (Research Mixt Unit representative), André Rouzière (CIRAD-cp, Head of coconut research program),

9h45-10h: coffee break (room 151)

10h-11h45 (room 151)
3- **Short presentation by participants:**

- **10h-10h15** : Presentation of IRD (JC Prot director or/De Noni IRD co-director, and Valérie Rotival, Public relation and Communication Officer),

- **10h15-10h30** : Presentation of Research Mixt Unit (Yves Duval / Françoise Dosba),

- **10h30-10h45** : Presentation of coconut programme developed by Cirad (André Rouzière, CIRAD-cp, coconut

- **10h45-11h05; 11h05-11h15; 11h15-11h35; 11h35-11h55-**
Presentation by the welcomed trainees of their own Institutes and research programme,

11h55-12h30
4- **Visit of the IRD Laboratories** (Valérie Rotival)

12h30-14h
5- **Lunch at IRD with all the participants: Room 151**

Afternoom: -

1- Presentation of the coconut biotechnology programs developed at IRD by Valérie Hocher and Jean-Luc Verdeil.

14h-14h30: **Jean-Luc Verdeil** (Somatic embryogenesis), (room 151)
14h30-15h: **Valérie Hocher** (cellular cycle) (room 151)

15h-15h30 (room 151)
2- Presentation by Florent Engelmann of cryopreservation generalities,

15h30-16h (room 151)
3- Presentation of Oulo'works on coconut cryopreservation of complete zygotic embryo and plumule
Part one: the big lines of Oulo's work developed on coconut.

16h-17h30 (Tissue culture laboratory)

Tuesday 14th October:

Morning: (Tissue culture laboratory)

Afternoom: (Tissue culture laboratory)

Wednesday 15th October:

Morning: (Tissue culture laboratory)

Afternoom:

14h-14h45 (Room 151)
1- **Presentation of Oulo'works** on coconut cryopreservation of complete zygotic embryo and plumule. **Part two**: A more adapt presentation to answer point to point to the different question which could appear after these first days training.

2-. (Tissue culture laboratory)

3- (Tissue culture laboratory)

Thursday 16th October:

Morning: (Tissue culture laboratory)

Afternoom: (Room 108)

14h-15h
1- Presentation with Jean-Luc Verdeil of the different steps of histology, with the observation of the corresponding slides for the treatments used (sucrose/dehydration/cryopreservation).

15h15-15h45
2- Presentation by Jean-Luc Verdeil of the "Plant histo-cytology and cell imaging unit"

Friday:

Morning: (Room 151)

1- Meeting of people involved in cryopreservation and dehydration aspects at IRD,

9h15
- **Stéphane Dussert**: *The value of using DSC (Differential Scanning Calorimetry) analysis in seed cryopreservation studies.*

10h → Coffee break

10h15-11h
- **Frédérique Aberlenc**: *Physiological and molecular basis of the desiccation tolerance in palm oil zygotic and somatic embryos,*

2- Short presentations on oil palm biotechnologies:

11h-11h30
- **Yves Duval** (Biology of the development of cultivated perenial tropical plants group head, IRD)

11h30-12h
- **Alain Rival** (Head of Oil Palm Program, CIRAD)

12h-12h30
3- Meeting of CIRAD researchers involved on coconut (to be define with A. Rouzière).

Afternoom up to 15h30/16h30: (Room 151)

14h-14h30
1- Conservation strategies (Florent Engelmann),

14h30-15h00
2- Presentation of action plans by participants,

15h00-15h15
3- Evaluation of training course,

15h15-15h45
4- Problem solving, conclusion of the training

15h45-16h00
5 - Closing ceremony and delivery of training certificates.

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Biologie du développement des Espèces Pérennes Cultivées

Summary of activities of the Joined Research Unit 1098 : "Developmental Biology of Perennial Crop Plants" (UMR BEPC)

This Joined Research Unit, which is located on 3 different sites in Montpellier (France) consists of 3 groups:
- The AFEF (Fruit Tree Architecture and Function) group on the ENSA.M-INRA campus, consisting of staff members from ENSA.M and INRA
- The BDPPT (Developmental Biology of Tropical Perennial plants) group at IRD, consisting of staff members from IRD, CIRAD and University Montpellier II
- The BGPT (Tropical Plant Biology and Genomics) group at CIRAD, consisting of staff members from CIRAD, INRA and University Montpellier II.

The **developmental biology** of the chosen perennial crop plant species is being studied from various different angles, including the molecular, cellular, organ and whole plant levels. In terms of the plant's life cycle, research themes tackled by members of the Joined Research Unit start at the **embryogenic (somatic) cell**, progressing to the **zygote**, then the juvenile plant and finally to the sexually mature **adult plant**. One of the original aspects of **perennial plants** is that an individual may live for many decades and modify its development throughout its lifetime.

The bulk of knowledge acquired within the scientific community to date on plant developmental biology relates to annual plant species. Our Joined Research Unit seeks to take advantage of generic data obtained with **model plants** such as *Arabidopsis* and rice in order to obtain an in-depth understanding of key developmental processes in **perennial crop** species. The main themes covered are:
- somatic embryogenesis
- somaclonal variation and molecular basis of flowering
- rhizogenesis and symbiosis
- plant architecture and lignification

The main species studied are crop plants of both temperate and tropical regions: **apple**, **coffee**, **filao tree** and **oil palm**. Other species such as apricot, rubber tree, coconut, olive and vine are also used to study specific processes of interest.

The BEPC Joined Research Unit forms part of the Plant Genomics and Integrative Biology Federative Research Institute (IFR 127 GBIP), which groups together 5 different Joined Research Units. One of main contributions of the BEPC Joined Research Unit to the GBIP Federative Research Institute is the provision of a cell imaging laboratory.

The BEPC Joined Research Unit also participates in the **Montpellier Integrative Biology Doctoral School** and conducts the training of Masters and PhD students within its research laboratories. Post doctoral fellows are also accommodated in the Joined Research Unit, in particular foreign nationals who work in cooperation with CIRAD and IRD.

The BEPC Joined Research Unit also provides an important contribution to the development of Montpellier's international teaching and research centre Agropolis, as well as to teaching activities at Montpellier Agriculture School and the University of MontpellierII.

Training in research is carried out by the Joined Research Unit through its playing host to numerous students for the work placement component of their Higher Education studies. Training is also given to visiting scientists from France or other countries in order to develop new skills and collaborations.

Plant cryopreservation: progress and prospects

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Paper presented during the IPGRI-COGENT/IRD Coconut Cryopreservation Training Course, IRD Montpellier, France, 13-17 October, 2003.

Cryopreservation (liquid nitrogen, -196 °C) represents the only safe and cost-effective option for long-term conservation of germplasm of non-orthodox seed species, vegetatively propagated species and of biotechnology products. Classical cryopreservation techniques, which are based on freeze-induced dehydration, are mainly employed for freezing undifferentiated cultures and apices of cold-tolerant species. New cryopreservation techniques, which are based on vitrification of internal solutes, are successfully employed with all explant types, including cell suspensions and calluses, apices, somatic and zygotic embryos of temperate and tropical species. The development of cryopreservation protocols is significantly more advanced for vegetatively propagated species than for recalcitrant seed species. Even though its routine use is still limited, there are a growing number of examples where cryopreservation is employed on a large scale for different types of materials, including orthodox and intermediate seeds, dormant buds, pollen, biotechnology products and apices sampled from *in vitro* plantlets of vegetatively propagated species. Cryopreservation can also be employed for uses other than germplasm conservation, such as cryoselection, *i.e.* the selection through freezing of samples with special properties, or cryotherapy, *i.e.* the elimination of viruses from infected plants through apex cryopreservation. Because its high potential, it is expected that cryopreservation will become more frequently employed for long-term conservation of plant genetic resources.

The value of using DSC analysis in seed cryopreservation studies

S. Dussert, IRD

Paper presented during the IPGRI-COGENT/IRD Coconut Cryopreservation Training Course, IRD Montpellier, France, 13-17 October, 2003.

Based on the results obtained with soybean, pea (Vertucci, 1989) and coffee seeds (Dussert *et al.*, 2001), when using low to high cooling rates (1 to 200°C.min^{-1}), the higher limit (*HL*) of the hydration window for cryopreserving whole seeds of lipid-rich species corresponds to their unfreezable water content. In non-orthodox oily seeds, the *HL* may also correspond to the optimal hydration level for whole seed cryopreservation, as shown with all coffee species studied, and, in some species (e.g. *C. arabica*), it is even the only hydration level at which seedlings can be recovered from cryopreserved seeds. Thus, determining the unfreezable water content of seeds of a given species by DSC analysis prior to cryopreservation trials maximizes the chances of achieving successful cryopreservation. For pea, soybean and coffee seeds, the water activity corresponding to the seed unfreezable water content is comprized between 0.75 and 0.85. Therefore, if non-orthodox seeds are desiccated by equilibration in a controlled atmosphere, testing a limited number of relative humidities between 75 and 85% should allow reaching directly this optimal hydration level for whole seed cryopreservation. It has been shown that the unfreezable water content of coffee seeds is negatively correlated with their lipid content. Therefore, measuring the lipid content of seeds to be cryopreserved constitutes an alternative method to estimate, in terms of water content, the hydration level at which seeds are amenable to cryopreservation. It should be noted that the fact that seeds tolerate desiccation to their unfreezable water content is not a sufficient condition for them to tolerate exposure to LN temperature after desiccation to this water content.

Text reproduced from "Dussert S., Chabrillange N. and Engelmann F. 2003. Determination of the hydration window for cryopreservation of intermediate oily seeds. In: Pritchard H. (Ed) Seed conservation: turning science into practice. Royal Botanic Gardens, Kew: in press".

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Physiological and molecular basis of desiccation tolerance in oil palm zygotic and somatic embryos

Frédérique Aberlenc-Bertossi

Paper presented during the IPGRI-COGENT/IRD Coconut Cryopreservation Training Course, IRD Montpellier, France, 13-17 October, 2003.

Desiccation is a natural event occurring at the end of development of orthodox seeds, i.e. seeds that tolerate dry and cold storage (Roberts, 1973). In contrast, recalcitrant seeds are generally damaged by dehydration and cannot be stored for extended periods. Various processes have been mentioned in connection with the acquisition of desiccation tolerance (reviewed by Pammenter and Berjak, 1999). Specific compounds such as heat stable and LEA proteins and soluble sugars have been proposed as protective substances especially in the dehydrated state. Desiccation tolerance is acquired during late embryogenesis in seeds still on the mother plant and is lost after germination. The main regulatory factors through which the seed environment controls embryo development are thought to be abscisic acid (ABA) and restricted water uptake.

Oil palm (*Elaeis guineensis* Jacq.) seeds can be stored for 2-3 years, and isolated oil palm embryos can tolerate desiccation (Grout *et al.*, 1983). However, given the reduced viability at cool storage temperature, oil palm seeds appear to display an intermediate storage behaviour, being neither orthodox nor recalcitrant (Ellis *et al.*, 1991). Somatic embryos, produced from embryogenic suspension cultures, exhibit morphologic similarities with their zygotic counterparts (Aberlenc-Bertossi *et al.*, 1999). However, they accumulate poor storage products and are sensitive to desiccation which limits the storage possibilities. To improve our knowledge of the biology of oil palm embryo development and factors involved in desiccation tolerance, late events occurring during zygotic and somatic embryogenesis were inverstigated. Changes in sugar, dehydrin-like proteins and abscisic acid (ABA) content, were particularly studied.

Throughout the zygotic embryo development *in planta*, acquisition of desiccation tolerance was positively correlated with embryo age and dry weight, and negatively correlated with initial water content during this period (Aberlenc-Bertossi *et al.*, 2003). Sucrose, the main soluble sugar present throughout embryo development, accounted for an average of 24% of the dry weight. Glucose and fructose contents decreased to less than 1 mg g^{-1} DW in embryos at maturity. At 117 DAP, as embryos became tolerant to desiccation, the monosaccharides/sucrose ratio fell to 0.015 and raffinose was detected. Stachyose appeared later in 147-day-old embryos and accumulated until shedding. Changes in the levels of abscisic acid (ABA) was studied throughout zygotic embryo development and a peak of accumulation of ABA was detected. The increase in ABA content was concomitant with embryo accumulation of dry matter, dehydration and acquisition of desiccation tolerance. Furthermore, storage proteins, identified as globulins, and dehydrin-like protein were accumulated during embryo development.

Somatic embryo desiccation tolerance, very low in standard culture conditions, was increased by sucrose and ABA supply (Aberlenc-Bertossi *et al.*, 2001). Exogenous sucrose accumulated in somatic embryos and induced a fall in the monosaccharides/sucrose ratio but neither raffinose nor stachyose was detected. ABA levels in somatic embryos was 2500 times lower than the maximum endogenous contents found in the zygotic embryos developed *in planta*. ABA supply induced an increase in ABA contents in somatic embryos which strongly suggest that the exogenous ABA penetrated in the tissues and accumulated there. However, the high percentages of ABA-glucose ester which represented 85.9% of total ABA in embryos cultivated on a medium enriched with ABA, suggested that a significant part of the ABA supplied was glycosylated by the tissue. Furthermore, ABA stimulated the synthesis of

raffinose and dehydrin-like protein in somatic embryos. Both Sucrose and ABA treatments favoured deposition of storage proteins and were found to stimulate maturation and to inhibit precocious germination of somatic embryos. However, sucrose and ABA supply during somatic embryogenesis were not able to induce a maturation as complete as during zygotic embryogenesis.

Proteases, involved in the mobilisation of storage proteins during germination (Callis, 1995), were studied in oil palm embryos. Proteinases activity, specific of zygotic embryos germination, were inhibited during maturation *in planta* thus allowing accumulation of storage proteins. On the other hand, proteinases activity found during *in vitro* development of somatic embryos could be partly responsible of the lack of protein observed in these embryos and consolidate the hypothesis of their precocious germination.

Recently, key genes involved in the regulation of maturation have been identified in model plants. *ABI3*, *FUS3* et *LEC1* may play a major role by stimulating maturation processes and by repressing simultaneously germination (Koornneef *et al.,* 1984 ; Kurup *et al.*, 2000). Numerous genes are expressed in response to environmental stresses such as drought and cold. Some of them encode protein factors that are involved in further regulation of gene expression and signal transduction and that function in stress response (Shinozaki et Yamaguchi-Shinozaki, 1997). Among them, *AREB, DREB* and *EREBP* have function respectively in response to ABA, dehydration and ethylene. The characterization of these genes have been undertaken in oil palm in order to determine their role in the orientation of embryo development towards maturation or germination and in the acquisition of desiccation tolerance.

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DNA Methylation *vs.* Somaclonal Variation in Higher Plants: Oil Palm as a Case Study

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Keywords: Elaeis guineensis *Jacq, epigenetics, genetic fidelity, somatic embryogenesis*

In the aim of understanding molecular phenomena underlying the « mantled » somaclonal variation in oil palm, our research work has focused on the role of genomic DNA methylation. Two complementary methods, aimed at evaluating the methylation rate at the genome-wide scale, have previously demonstrated the occurrence of a highly significative DNA hypomethylation in leaves and calli from abnormal regenerants, compared to their normal counterparts. Investigations are now aimed at targeting the individual sequences which epigenetic misregulation could account for the onset or for the maintenance of the « mantled » phenotype.

We are currently trying, through a wide range of techniques, to focus our methylation studies on the only genes which are relevant with regard to the « mantled » phenotype.

In this aim, we have undertaken methylation-sensitive RFLP and AFLP studies, involving the isoschizomeric enzymes *Msp*I and *Hpa*II. RFLPs were envisaged primarily in order to screen a pool of oil palm cDNA, while the aim of MSAP (methylation-sensitive amplified polymorphism) investigations was to generate a large number of relevant markers, exhibiting a differential methylation pattern depending on the normal/mantled phenotype but independent of the genetic origin of clones.

Hypomethylating drugs were used in embryogenic cell suspensions in order to evaluate the impact of DNA demethylation on the genotype of regenerants and to simultaneously assess the set of methylation-sensitive markers previiously developed.

By studying oil palm DNA-methyltransferases, it will be possible to determine whether DNA methylation is the only epigenetic regulator affected, and which regions of the genome are specifically modified in variants.

Development of complementary strategies for plant genetic resources conservation

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It is now well recognized that, for any given genepool, a number of different and complementary approaches and methods are necessary for efficient and cost-effective conservation. Two basic conservation strategies, each composed of various techniques, are employed to conserve genetic diversity, *i.e.*, *in situ* and *ex situ* conservation. *Ex situ* conservation means the conservation of components of biological diversity outside their natural habitat. The *ex situ* conservation methods include storage in seed genebanks, field genebanks, *in vitro* genebanks (including slow growth storage and cryopreservation), and DNA banks. *In situ* conservation means the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties. *In situ* conservation methods include nature reserves, managed areas and farmers' fields. The following key parameters should be considered when planning complementary conservation strategies. The extent of the genepool coverage and the distribution of genetic diversity should be assessed, both within the genepool and geographically. The reproductive biology of a species is critically important to decide which methods are applicable. The extent of genetic erosion and other threats need to be taken into consideration. The nature of the plant material to be stored, together with the importance of a continued evolution for the germplasm, are also important considerations. Furthermore, the socio-economic feasibility, the availability of human and financial resources, of conservation facilities and institutions, are other aspects to consider when deciding on the combination of available conservation methods.

CLONAL PROPAGATION OF COCONUT IN IRD-CIRAD.

IRD/CIRAD team : V. Hocher, J-L Verdeil

Paper presented during the IPGRI-COGENT/IRD Coconut Cryopreservation Training Course, IRD Montpellier, France, 13-17 October, 2003.

Coconut clonal propagation could allow the multiplication of palms selected for their resistance or tolerance to various diseases and pests. Since almost 20 years, the CIRAD has a joined research program with IRD for coconut clonal propagation through somatic embryogenesis. This program was shown to be a very difficult challenge due to the extreme recalcitrance of coconut tissues to *in vitro* culture[1]. The main steps of coconut multiplication by somatic embryogenesis are now well mastered (i.e. callogenesis, somatic embryogenesis induction, development of complete somatic embryos) and the team is actually having a protocol which allow a mean production of 12 to 20 plantlets/year/genotype.

Mass propagation which can ensure a high scale production of clonal plantlets coming from a selected individual is still limited due to a reduced development of the vitroplantlets. Indeed, even if somatic embryos are regularly obtained, their germination and further development into plantlets remains extremely difficult. Studies on physiology[2] and histology[3] of coconut vitroplantlets have revealed that the problem was link to the length of morphogenesis events in this species and to the rapid lost of meristematic potential of the tissues cultivated *in vitro*. Thus a slow growth of the plantlets leaves, a high chronaxy, a very weak mitotic index and an important chromatin condensation of the cells nuclei (characteristic of quiescent cells) were observed in *in vitro* neoformed tissue, leading to the hypothesis of a slowing down and/or a jamming of the cell cycle in this tissues.

In 1999, the IRD/CIRAD group has started a study of cell cycle in coconut. Studies using flow cytometry revealed that the majority (>90%) of the meristematic cells of different tissues (calli showing either a rapid (CCR) or a slow growth (CCN), caulinary apex, immature leaves) were in G0/G1 phase of the cell cycle[4]. In plants, few studies have been conducted on G0/G1 cells and their re-entry in the cell cycle. On the opposite this phenomenon is well known in animals as the re-activation of quiescent cells towards a cycling state is one of the sign of cancerisation. The numerous studies have revealed the predominant role of the retinoblastoma protein pathway[5], which implies different genes : Rb protein gene, E2F a transcription factor, cyclin D;... Recently, homologous of some of these genes have been isolated in different plants species[6]

On the base of these knowledge, the IRD/CIRAD team has started the search for genes implicated in the coconut cell cycle regulation. Heterologous probes (Maïs, Tobacco,...) have been used to screen caulinary apex cDNA libraries. Four full length cDNA have been isolated and sequenced, revealing high homology with different genes of the retinoblastoma pathway : cyclin D2, E2F, CDC2 and Rb. It seems thus that this way of cell cycle regulation is present in coconut. The team is actually focusing the work on the expression characterisation of these genes in different types of tissues cultivated under various culture conditions.

The next step of this program will be to understand how the retinoblastoma pathway could control the expression of the meristematic potential in coconut tissue cultivated *in vitro* and how this expression can be modulated. This requires different types of approaches. A study at the protein level will be initiated. The phosphorylation profiles of the retinoblastoma protein will be analysed by immunological techniques (Western blot, Immunolocalisation) in different coconut tissue cultivated under various conditions. The formation of the protein complex E2F-Rb (known as the key point in the transition between G0/G1 phase and S phase of the cell cycle) will be study *in vitro* (double hybrid technique, immunoprecipitation) and *in situ* (FRET). The search for genotypes associated to an overexpression of Rb and E2F genes will also be started using heterologous system (Rice, Arabidopsis).

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Presentations occurred during the *IPGRI-COGENT/IRD* Coconut Cryopreservation Training Course, IRD Montpellier, 13-17 October 2003
B. Malaurie, Coordinator

Cryopreservation of coconut (*Cocos nucifera* L.) caulinary and/or zygotic embryos for a safe conservation and transfer of plant material free of diseases

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Abstract:

The coconut is a plant with recalcitrant oleaginous seed, one of the largest of the plant kingdom with non dormant embryo. Most of coconut conservation is realised in field in regional collections, where risks of genetic erosion are important, because of floods, pests and diseases. Since the 1980s impact of coconut diseases are increasing with viral, viroid and mollicute diseases, where for the last one, the Lethal yellowing (LY) is at the origin of a heavy lost. On account of the nut size, the lack of dormancy of the embryo, the risk of complete destruction of coconut plantation by LY expansion, this conservation mode appears to be problematic for the conservation of coconut genetic resources, and makes germplasm exchanges between producer's countries uneasy.

In front of these problems, *in vitro* culture and overall conservation through cryopreservation appear as a complementary way which can help to resolve them. Since previous works on cryopreservation of complete coconut zygotic embryo in 1992, it appeared that the technique was applicable to coconut with interesting results. However it was observed that only meristematic tissue survived to freezing. *In vitro* culture of plumule (caulinary meristem with one to three primordia) appeared then to be an attractive starting material for cryopreservation as it is known to be free from viral diseases, and as it make possible the use of more efficient cryopreservation technique like encapsulation-dehydratation.
In this context cryopreservation using plumule was undergo through encapsulation-dehydration technique, where recovery of plantlet was obtained after freezing. In parallel, validation of the cryopreservation process of complete mature zygotic embryos through the largest diversity representative of all the different areas of coconut culture was developed, and it good performance confirmed.
To confirm the safety of the exchange of coconut though zygotic embryo form or plumule form, LY-like infected zygotic embryos, were collected from LY-like infected Ghanaian area. Research of the presence of the phytoplasma, infectious agent of LY disease, inside of the tissue of embryo was performed by PCR by the amplification of the 16S rDNA sequence characteristic of the phytoplasma. Valuable results have been obtained and interpretation undergoing.

The Role of Embrapa in the Brazilian Agricultural Research Development

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The Brazilian Agricultural Research Corporation's mission is to provide feasible solutions for the sustainable development of Brazilian agribusiness through knowledge and technology generation and transfer. From the very beginning, in April 26, 1973, Embrapa has generated and recommended more than nine thousand technologies for Brazilian agriculture, reduced production costs and helped Brazil to increase the offer of food, at the same time, conserving natural resources and the environment and diminishing external dependence on technologies, basic products and genetic materials. Embrapa was founded and implanted with the support of international cooperation. From the start, the Corporation counted on the collaboration of international and foreign organizations, Universities, the World Bank (BIRD), the Inter-American Institute for Cooperation on Agriculture (IICA), the Inter-American Development Bank (IDB), all of which continue as outstanding collaborators up to the present.

Embrapa is organized as a large network, composed of 40 decentralized Centers distributed among the several regions of Brazil. Networking through 37 Research Centers, 3 Service Centers and 11 Central Divisions, Embrapa is present in almost all the states of the Union, each with its own ecological conditions. There are 8,619 employees in Embrapa, of which 2,221 are researchers, 45% with master's degrees and 53% with doctoral degrees. Embrapa coordinates the National Agricultural Research System, which includes most public and private entities involved in agricultural research in Brazil.

Embrapa is not only the largest Agricultural Research Institution in Brazil, but extends its actions to North America and Europe as well, where it maintains two laboratories of technological prospecting and institutional exchange in the United States (Labex US) and France (Labex France). Embrapa maintains projects in International Cooperation in order to perfect knowledge of technical and scientific activities or to share knowledge and technology with other countries. Embrapa's growing participation in the international scene, and perspectives for its enlargement at an ever increasing rate in view of globalization and international economic integration, represent a decisive invitation for Embrapa to play an active role in the transfer of agricultural science and technology at the international level.

The Corporation is today recognized internationally as possessing a significant and qualified reserve of scientific and technological knowledge, especially for tropical regions. With these credencials, Embrapa has been functioning as a provider of technical-scientific cooperation to other countries.

Embrapa develops special programs and projects concerning areas such as food safety, family agriculture, natural resources, advanced technology and agribusiness, and acts as a partner in several others. Between your technologies stand outs: Agricultural Instrumentation, Agroindustry, Animal Feed, Animal Health Sanitary Control, Animal Production, Biologycal Control, Biotechnology, Forest Resources, Fruits and Vegetables, Grains, Information Products, Information Science, Natural Resources and Environment, Raw Materials and Root and Tubers.

The Embrapa Genetic Resources & Biotechnology is a Embrapa's national reference center for biotechnology researches . Its mission is make possible technological solutions for the maintainable development of the Brazilian agribusinees, assuring the conservation and use of the genetic resources, generating, adapting and transferring knowledge and technologies, in benefit of the society. The major research groups are Biotechnology, Genetic Resources, Biological Control and Biological Security.

The Embrapa Coastal Tablelands is a research center studies the social-economic and natural resources of the Coastal Tableland, an ecosystem 30 to 150 meters above sea level, which extends from the state of Amapa, in the extreme North of Brazil to the state of Rio de Janeiro, in the Brazilian Southeast. The center works in collaboration with other research institutions and develops activities and monitoring the Mata Atlântica (Atlântica forest) in the Northeast. It is a national reference for research in the improvement, management and phytosanitary control of the coconut. With the support of international institutions, it maintains the biggest coconut germplasm bank in South America.

The major research areas are Natural Resources and Social-economical Zoning, Monitoring of the Remainder of the Mata Atlantica (Atlantic forest) , Recovery of Degraded Pastures and Obtaining of Coconut Hybrids, Phytosanitary Protection and Biotechnology (Tissue Culture and Molecular Biology).

In the biotechnology area the main reaserch lines are: Coconut (*Cocos nucifera* L.), Mangaba (*Hancornia speciosa* Gomes), a indigenous fruit, and Neem tree (*Azadirachta indica* A. Juss) micropropagation; Coconut embryo *in vitro* culture; Insert of genes for resistance to coconut diseases; DNA marker for characterization of genome; Molecular detection and diagnosis of pathogens and Use of molecular markers as soil bioindicator. Actions of future researches: *In vitro* conservation of coconut genetics resources and indigenous fruits.

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COCONUT RESEARCH PROGRAMME AT CICY

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The Centro de Investigación Científica de Yucatan (CICY) in Merida (Mexico), is a research institute focused on the study of tropical crops biology and biotechnology. CICY is linked to the Consejo Nacional de Ciencia y Tecnología (CONACYT) and It is divided in four research units: Biotechnology; Molecular Biology and Biochemistry; Natural Resources; and Material Sciences.

Research on different crops is carried out within Institutional research programs based on intra- and inter-institutional collaboration. Such is the case of the coconut research programme that is mostly dedicated to the study of the lethal yellowing (LY) disease and how to deal with it. This disease is one of the most pressing problems associated to this crop in Mexico since It has devastated most coconut plantations in the Yucatan Peninsula and the Gulf of Mexico coasts. The main objectives of the programme are: (a) to carry out research to characterize the genetic diversity of coconut germplasm (including LY resistance), in order to facilitate coconut conservation and improvement; (b) to develop *in vitro* culture protocols for coconut propagation and safe movement; and (c) to study the LY plant-pathogen-vector-environment relations in order to improve the control of the disease.

Since the most effective way to control LY is replanting with resistant palms, CICY has established a germplasm collection / resistance trial that has evaluated most of the coconut germplasm present in Mexico, mostly tall genotypes from the Pacific coasts, during more than 10 years now. Several of these genotypes have shown high levels of resistance to LY. They have been characterized morphologically and biochemically, and molecular characterization is currently in progress in collaboration with CIRAD (France). These genotypes have been used to develop hybrids dwarf x tall and tall x tall in collaboration with INIFAP (Mexico), and field trials of these materials are currently carried out. In order to propagate the resistant materials, CICY in collaboration with Wye College (UK), IRD/CIRAD (France) and more recently with INIFAP (Mexico) developed a micropropagation protocol using plumule explants based on somatic embryogenesis capable of producing over 500 somatic embryos per zygotic embryo. Plantlets obtained can be successfully transferred to *ex vitro* conditions. In order to try to increase the efficiency of the process, studies to characterize some of the molecular regulatory mechanisms being present during somatic embryo formation are carried out. In addition, research is also carried out to develop methods for molecular improvement. A collaboration effort between researchers from Max Planck and Fraunhofer institutes (Germany), Florida University and CICY is focused on developing transformation protocols for the introduction of resistant traits in coconut lines.

Regarding the disease, collaborative research is been carried with Colegio de Posgraduados and INIFAP (in Mexico) and the University of Florida (USA) and Rothamsted Research (UK), and more recently Zamorano (Honduras), Universidad del Valle (Guatemala) and IIFT (Cuba), on the development of methods of sampling and detection of the LY pytoplasmas, phytoplasma diversity, plant pathogen relations, transmission and epidemiology. Methodology has been developed for sampling and detection, and for eradication based on the epidemiological studies, and both are currently being used. Monitoring of the geographic spread of LY is being carried within the Latin America and the Caribbean region, and several strains of LY phytoplasmas have been identified, some of these coexisting with the resistant varieties on the Pacific coasts of Mexico. Research has also contributed to increase the knowledge of plant-pathogen relations and a very comprehensive model has been produced. Overall, research has already yielded results that have allowed contributions on genetic control, quarantine and eradication.

Mikocheni Agricultural Research Institute
The Institute's Profile and its Research Programme

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Abstract

In Tanzania, the coconut palm (*Cocos nucifera* L.) is an important perennial cash and subsistence oil crop in the coastal belt. It supports the livelihood of more than 300,000 households who cultivate about 25 million palms on approximately 252,000 ha.
During the 70's it became apparent that coconut production and productivity was declining due to a number of constraints. In 1979/80 the Government of Tanzania established the National Coconut Development Programme (NCDP) with the objective to reverse the state of declining production through various R&D activities. It is now being implemented by Mikocheni Agricultural Research Institute (MARI).
One of the targets of the National Development Vision 2025 is to reduce by 50% the number of people living in abject poverty by the year 2015 and to eradicate poverty by year 2025. To realize this Vision Tanzania considers science and technology (S&T) to play a central role. It is against this background that the Ministry of Agriculture and Food Security (MAFS), through the Tanzania Agricultural Research Programme – Phase II (TARP II) has decided to take an initiative of strengthening its agricultural biotechnology research and development capacity, which is generally low due to human and infrastructure limitations. In the shot term, emphasis will be to develop the capacity in tissue culture and micro propagation techniques at a number of its Agricultural Research Institutes (ARIs). The research programme of MARI has, therefore, two components, i.e. coconut and biotechnology research sub-programmes.

Coconut Research in PNG

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Abstract

The PNG Cocoa and Coconut Research Institute (PNGCCRI) established since 1986 is mandated to conduct all research work on cocoa and coconut in the country. PNGCCRI is the research entity of the Cocoa and Coconut industries in Papua New Guinea. The cocoa and coconut are under separate organizations governed by the Board of Directors.

The coconut research in PNG only intensified into a more systematic approach since the establishment of CCRI in 1986. Research under the then Department of Agriculture conducted experiments on nutrient requirements of the crop and this has shifted to breeding research in the late 1970s which promoted the introduction of the MAREN hybrids for the major replanting program in the country.

In the later developments, COGENT has facilitated in the promotion of coconut genetic improvement program since its inception in 1994. The major can has been the set up of the ICG for the South Pacific. Other organisations such as CIRAD and ACIAR have contributed in coconut research in PNG under the specified projects.

The major under taking in the genetic improvement program is initially the development of the genebank and the planned introductions of the proposed 200 selected varieties from the participating countries. In order to accomplish this, COGENT is currently facilitating the development of the genebank by providing some technical and financial support. There are also other sources of germplasm within the country from which the ICG-SP can collect for conservations and utilization.

Determination of the hydration window for cryopreservation of intermediate oily seeds

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Poster from "Seed Conservation: Turning Science into Practice, Royal Botanic Garden, Kew, 27-30 July 2001"

Introduction. Cryopreservation is the only technique available for long-term conservation of genetic resources of non-orthodox seed species. In order to determine the limits of the hydration window for cryopreservation (HWC) of intermediate oily seeds, the effect of exposure to liquid nitrogen (LN) temperature on viability of seeds desiccated to various water contents was investigated in nine coffee species previously shown to display a high variability in seed desiccation tolerance [1].

Results and Discussion (1). Three groups of species could be distinguished based on seed survival after LN exposure (Table 1). In group 1 species, no seedling production could be obtained after LN exposure. In group 2 species, recovery was very low or nil after rapid cooling and only moderate after slow cooling (see Fig. 1 for *C. eugenioides*). In group 3 species, very high percentages of normal seedling development were observed after both rapid and slow cooling. When expressed in terms of water content, the higher limit of the HWC (Fig. 1) was highly variable since it ranged from 0.14 to 0.26 g H_2O g^{-1} dw (g/g) (Table 1). In group 3 species, the sensitivity to LN exposure was due to endosperm injury as shown by the development into normal seedlings of embryos extracted from seeds after thawing (see Fig. 2 for *C. stenophylla*).

Table 1. Desiccation sensitivity, as estimated by the water content at which 50% of the initial viability was reached, WC_{50}, higher limit of the hydration window for cryopreservation, *HL*, percentages of normal seedlings recovered after desiccation to *HL* and direct immersion into LN (rapid cooling) or by a precooling to -50°C at 1°C.min^{-1} prior to immersion in LN (slow cooling), unfreezable water content, WC_u, and corresponding water activity, a_{wu}, of seeds of nine coffee species classified in three groups according the percentages of normal seedling recovery after LN exposure (g/g = g H_2O g^{-1} dw; ND = not determined).

Group	Species	WC_{50} (g/g)	*HL* (g/g)	Normal seedlings (%)		WC_u (g/g)	a_{wu}
				Rapid cooling	Slow cooling		
1	*C. brevipes*	0.20	-	0	0	ND	ND
	C. canephora	0.17	-	0	0	0.28	0.86
	C. liberica	0.29	-	0	0	0.26	0.85
	C. stenophylla	0.16	-	0	0	ND	ND
2	*C. arabica*	0.11	0.21	0	17	0.21	0.78
	C. eugenioides	0.11	0.26	8	19	0.26	0.86
3	*C. pseudozanguebariae*	0.06	0.14	73	68	0.14	0.75
	C. racemosa	<0.14	0.23	67	73	0.24	0.83
	C. sessiliflora	<0.14	0.19	81	76	0.18	0.79

Results and Discussion (2). With all species of groups 2 and 3, the higher limit of HWC corresponded also to the optimal hydration level for seed cryopreservation (see Fig. 1 for *C. eugenioides*). In those species, a highly significant correlation was found between the unfreezable water content, as determined from DSC analysis [2], and the higher limit of the HWC (Fig. 3). This result is consistent with the earlier study of Vertucci [3] with orthodox soybean seeds, and suggests that intracellular ice formation is lethal in lipid-rich seeds, independent of their level of desiccation sensitivity.

The unfreezable water content was negatively correlated with seed lipid content (Fig. 4). However, this relationship was not linear, even when expressed on a non-lipid dry weight basis, indicating that compounds other than lipids influence the unfreezable water content.

When expressed in terms of water activity, the interspecific variability for the higher limit of the HWC was very low and independent of seed lipid content (Table 1). This result, in combination with the fact that the higher limit of the HWC was always the optimal hydration level for seed cryopreservation, suggests that desiccating seeds under 75-85% RH at 25°C allows to reach directly the optimal water content for intermediate oily seed cryopreservation. This hydration level could be slightly above the optimal level for minimizing seed deterioration at LN temperature [4]

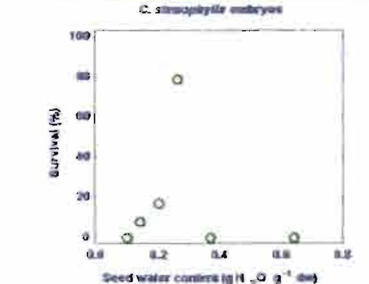

Fig. 2. Survival, (based on normal seedling development), of zygotic embryos extracted after thawing from *C. stenophylla* seeds desiccated to various water contents and frozen rapidly or slowly.

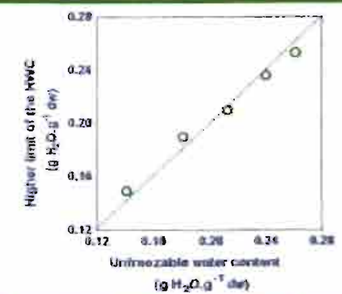

Fig. 3. Relationship between the unfreezable water content, WC_u, as determined from DSC analysis, and the higher limit of the HWC of seeds of the five species of groups 2 and 3, as determined in Table 1.

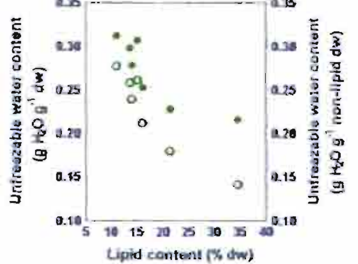

Fig. 4. Relationship between the lipid content and the unfreezable water content, expressed on a dry weight basis (●) or a non-lipid dry weight basis (○), of seeds of seven coffee species.

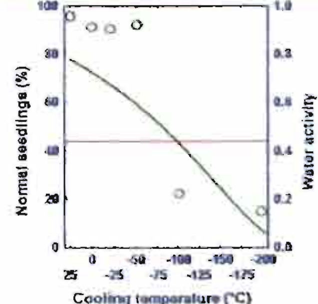

Fig. 5. Survival (○), water activity (—) and critical water activity for desiccation tolerance (—) of *C. arabica* seeds cooled to various temperatures after desiccation to 0.2 g H_2O g^{-1} dw. Seed water activity was calculated according to the van't Hoff isochore [4], with an enthalpy of sorption of -2 kJ.mol^{-1} H_2O at 0.2 g H_2O g^{-1} dw [5].

Results and Discussion (3). The lower limit of the HWC was always much higher than the critical hydration level for desiccation tolerance. Assuming that a single critical water activity determines the desiccation sensitivity of seeds of a given species, this result can be explained by the decrease in seed water activity with decreasing temperature. The relationship between the water activity, a_w, and the temperature, T (K), is described by the van't Hoff isochore [4]: $d(\ln(a_w)) / d(1/T) = \Delta H_{sorp(T)}/R$, where $\Delta H_{sorp(T)}$ is the enthalpy of sorption and R is the ideal gas constant. Assuming that ΔH_{sorp} is constant with temperature [4], the water activity of *C. arabica* seeds at 0.2 g/g was calculated for temperatures between +25 and -196°C using the value of ΔH_{sorp} at 0.2 g/g given by Eira *et al.* [5] (Fig. 5). When cooled to LN temperature, the water activity of seeds crossed the critical water activity for desiccation tolerance (0.45 [1]) at about -95°C. Interestingly, an abrupt decline in survival of *C. arabica* seeds at 0.2 g/g was observed between -50 and -100°C (Fig. 5). Therefore, the prediction of the existence of an hydration window for LN exposure in non-orthodox oily seeds should not be based on the comparison of the unfreezable water content with the critical water content for desiccation sensitivity, as measured at room temperature, but on the comparison of the critical water activity for desiccation damage, measured at room temperature, with the calculated water activity at LN temperature of seeds desiccated to their unfreezable water content.

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Nominations for IPGRI-COGENT funded participants to Coconut Cryopreservation Training Course at Institut de recherche pour le développement (IRD), Montpellier, France 13 - 17 October 2003

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