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CRYOPRESERVATION OF COCONUT ZYGOTIC EMBRYOS AND POLLEN

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Cryopreservation of Coconut Zygotic Embryos and Pollen

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Cover Front: A snap shot of activities involved in cryopreservation
Back: Plantlet retrieved from a cryopreserved embryo of Malayan Yellow Dwarf using modified pre growth desiccation

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Foreword

Cryopreservation is an important tool for the long-term storage of plant genetic resources since it requires only minimum storage space and enables easy maintenance. Plant Genetic Resources are the most valuable and essential basic raw material for any crop improvemet programme. Conservation is highly essential to safeguard genetic resources against genetic erosion, genetic vulnerability and genetic wipeout and sustainable agricultural production. In the present situation, with the emergence of new pests, diseases or crop production problems, the need for safe storage of all germplasm is emphasized as they may contain many valuable genes to tackle those problems. The coconut germplasm at present is being conserved in field gene banks. The major drawbacks of *ex situ* conservation in field gene banks are threat from pests, diseases, natural calamities, and high cost of maintenance. An alternative conservation strategy is the *in vitro* conservation which provides available back up to field gene bank.

The cryopreservation research in coconut was initiated at CPCRI in 2001 and the effectiveness of various protocols for successful recovery of plantlets from the cryopreserved embryos were evaluated. Among the four protocols tried at the biotechnology laboratory of CPCRI for cryopreservation of zygotic embryos, modified pre growth desiccation and use of PVS3 liquid were found to be the best. In these two methods, the plantlet recovery at the stage of field planting was observed to be 23 to 25%. The growth and reproduction of the plants derived from cryopreserved embryos were similar to that of normal plants derived from the seed nuts.

Coconut pollen is tricelled and generally shed at relatively high moisture content and can survive desiccation and thus can be cryopreserved without much difficulty. Cryopreservation of pollen is highly essential for conserving the nuclear genetic diversity. Pollen with distinguishable characteristics has great potential for future breeding programmes. This technical bulletin describes the steps involved in embryo and pollen cryopreservation.

I am sure that this technical bulletin "Cryopreservation of coconut zygotic embryos and pollen" will be of great benefit to the research personnel and students dealing with *in vitro* conservation.

October, 2010

(George V. Thomas)

Director

CONTENTS

1. Introduction

Plant Genetic Resources are the most valuable and essential basic raw material of any crop improvement programme and its conservation is highly essential for sustainable agricultural production as it safeguard against genetic erosion, genetic vulnerability and genetic wipeout. In the present situation of incidence of new pests, diseases or crop production problems the need for safe storage of germplasm is emphasized as they may contain genes to tacke those problems. The coconut germplasm at present is being conserved in field gene banks. The major drawbacks of *ex situ* conservation in field gene bank are threat from pests, diseases, natural calamities, and high maintenance cost. An alternative conservation strategy is the *in vitro* conservation which provides back up to field gene bank. Coconut embryos provide an ideal material of choice for collection and conservation of coconut genetic diversity (Karun *et. al.*, 1993, 2005; Sajini *et. al.*, 2006). Short to medium term storage of coconut zygotic embryos had been reported by Assy Bah and Engelmann (1992a and b) and Karun and Sajini (1994). For long term storage, cryopreservation or freeze preservation at ultra low temperature (-196°C) is the only safe and cost effective option available for the conservation of coconut genetic resources. Cryopreservation is based on non injurious lowering and subsequent interruption of metabolic functions of biological material by temperature reduction to the level of liquid nitrogen. Through cryopreservation the material can be stored for indefinite periods without loosing viability. The key element in tissue cryopreservation is the removal of intracellular water which becomes crystallized and cause damage or death of cells thereafter if not completely removed. The partial desiccation and freezing tolerance exhibited by coconut embryos is being utilized for cryopreservation studies (Assy bah and Engelmann 1992b). The cryopreservation studies in coconut started at CPCRI during 2001 and the effectiveness of various methods for successful recovery of plantlets from the cryopreserved embryos were evaluated thereafter.

Cryopreservation of pollen is highly essential for the conservation of nuclear genetic diversity (NGD) which is an efficient backup method for conserving genes (Towill and Waters, 2000). Coconut pollen is tricelled and generally shed at relatively high moisture content and survives desiccation. Coconut pollen viability at ambient condition lasts only for 6-7 days. By freeze preservation and storage at room temperature it is possible to conserve coconut pollen for short periods (Whitehead, 1966). Coconut pollen cryopreservation can be utilized for long term storage of pollen from palms of distinguishable characterstics for future breeding programmes, exchanging germplasm and for continuous availability for pollination over extended periods of time. The methodology for cryostorage of coconut pollen has been developed by Karun *et. al.* (2005).

The four different methods tried at CPCRI, Biotechnology laboratory for cryopreservation of coconut zygotic embryos and the protocol for coconut pollen cryopreservation were detailed in this Technical Bulletin.

2. Air Desiccation

Air desiccation is the simplest cryopreservation method that avoids the use of toxic penetrating cryoprotectants. It is being commonly used for desiccating excised zygotic embryos, embryonic axes, somatic embryos and embryogenic clumps of various plant species including recalcitrant seed species. The standardization of duration of desiccation is highly critical for the removal of water, which otherwise will contribute the formation of ice crystals in the intra cellular space when exposed to liquid nitorgen and will lead to death of the tissues. Whole plant recovery depends on critical moisture content that was found to be 20-23 % for coconut zygotic embryos. The two types of air desiccation pretreatments employed were the use of silica gel and air current of laminar flow. In coconut the optimum dehydration duration for maximum retrieval of healthy plantlets was found to be 18-20 hours (Moisture Content (MC) 20 % on fresh weight) using silica gel and 24-26 (MC 20 % on fresh weight) hours for laminar air current. When the water content of the embryo was reduced below 20 % desiccation damage resulted in death of the shoot meristem.

2.1. Checklist for desiccation method

Items needed for extraction and surface sterilization of embryos

i) Big knife
ii) Cork borer
iii) Small knife
iv) Beaker (100 ml, 1000 ml)
v) Tray
vi) Measuring cylinder
vii) Conical flask
viii) Sterile tools (Forceps)
ix) Sodium hypochlorite solution

Items needed for desiccating embryos

i) 250 ml wide mouthed bottle
ii) Silica gel
iii) Aluminium foil
iv) Tissue paper

Prepare in advance

i) Sterile water
ii) Sterile conical flask (100 ml)
iii) Retrieval medium - Y3 full strength medium (Annexure I) containing 40 g/l sucrose with activated charcoal 1g/l and Agar 5.8 g/l (pH 5.7)
iv) Sterile tissue paper, aluminium foil
v) Sterile cryovials

vi) Dehydrate silica gel (coarse type from Merck, India) by heating in a microwave oven till the colour become dark blue

vii) Fill 50 g of above in 250 ml bottle and autoclave

2.2. Procedure

Extraction of embryo




- ☐ Split open the nuts.
- ☐ Scoope out the embryo along with endosperm from beneath the large soft eye of the nut using a cork borer.
- ☐ Collect the endosperm plugs.
- ☐ Making a small incision in the endosperm with the help of a knife, etract the embryos from the endosperm and collect it in a beaker filled with distilled water

Surface sterilization of embryos

- ☐ Wash the embryos in distilled water two to three times to remove coconut milk and small pieces of endosperm.
- ☐ Put the embryos in a sterile conical flask (100 ml) inside the laminar air flow.
- ☐ Add 20% of sodium hypochlorite solution in sterile water and keep it for twenty minutes with occasional stirring.
- ☐ Drain off the sterilent and wash the embryos in sterile water 4 to 5 times

2.3.1. Desiccation of embryos in laminar air current

Place the surface sterilized embryos on a sterilized aluminium foil inside the laminar air flow chamber for a period of 26 hours

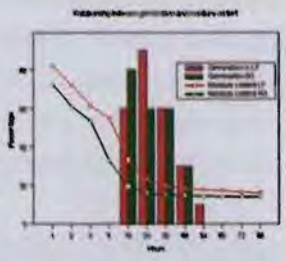
2.3.2. Desiccation of embryos using silica gel

Place the embryos (20 nos) over sterile activated silica gel inside a 250 ml bottle for 18 hours.

2.4. Determination of critical moisture content

In order to draw the standard desiccation curve to know the moisture content of the embryos after a given desiccation period, the initial weight of at least five embryos was recorded (F W), and thereafter weighed again for different desiccation periods (x), which is denoted by FWx. The embryo lots were then placed in an oven at 103°C for twenty four hours to extract all the remaining water and their dry weight (DW) was measured. By using the following formula the Moisture Content (MC) of the embryos were calculated after a given desiccation period (x) on fresh weight basis.

MCx = (FWx - DW/ FW) X 100



Moisture content obtained were plotted against different desiccation durations that enabled the drawing of standard desiccation curve which makes it possible to know the moisture content of a sample after a given desiccation period without having to weigh it during all future experiments. The critical moisture content was evaluated by correlating the moisture content corresponding to maximum percent germination.

2.5. Incubation in liquid nitrogen

In either case (silica gel or laminar airflow), put the dried embryos in sterile cryovials and directly plunge in liquid nitrogen.

2.6. Thawing

Take out the cryovials from the liquid nitrogen and put directly in water kept at 40°C for two minutes.

2.7. Retrieval of embryos

Incubate the embryos in full strength Y3 medium supplemented with 40 g/l sucrose and 1g/l activated charcoal in dark till the emergence of first leaf. In the case of tall varieties of coconut the germination starts within 30-35 days. Transfer the cultures to illuminated room with 16 hours photoperiod (temperature 27-29°C, relative humidity 65-70 %).

2.8. Sub culturing

Sub culture the embryos at monthly intervals into full strength solid Y3 medium supplemented with 4 % sucrose, 0.5 mg/l BAP and 0.5 mg/l NAA.

- Additional amount of BAP or NAA were added (BAP 2mg/l, NAA 5 mg/l) to in order to promote either shoot or root growth if it is not balanced.
- Sucrose concentration reduced to 3% after 3-4 subcultures.
- Subculture the plantlets into Y3 liquid medium in the later stages along with IBA 5 mg/l for better development of roots.

2.9. *Ex vitro* establishment

- Keep the plantlets (18-25 cm height) with balanced root (well-developed secondary and tertiary roots, 5-6 ml root volume) and shoot (3-4 leaves) system in Carbendazim (1 g/l) and IBA (1000 ppm) solution for 1 hour each.
- Transfer the plantlets to pots filled with sterilized sand, soil and coir dust in equal proportions.
- Cover the pots with polythene bags to maintain high humidity for initial establishment.
- Keep the plants in ambient condition under illumination.
- After two weeks make small perforations on the polythene cover to gradually reduce humidity.
- After two weeks remove the cover completely. Keep the plants with proper watering inside the lab for 1-2 weeks.
- Shift the potted plants to the green house under 50 % shade.
- Keep the plantlets in the nursery for two months.
- Transplant it to large polythene bag filled with soil and organic manure.
- Proper care should be taken for the healthy growth of plantlets by regular irrigation and manuring. Remedial measures are to be taken in case the plant is showing any disease symptoms.

3. Pre growth Desiccation

In desiccation based cryopreservation procedures, it is extremely desirable to induce high level of desiccation tolerance for survival after cryopreservation. When water is lost from a cell the membranes seems to be the primary site of injury. The role of sugars especially sucrose is the induction of desiccation tolerance and thereby protecting membranes after cryopreservation as it is confirmed in several plant species. The pre growth desiccation protocol developed at Biotechnology laboratory of CPCRI for coconut zygotic embryos involves extraction of embryos, surface sterilization, liquid nitrogen treatment, thawing, regrowth and *in virto and ex virto* establishment.

Prepare in advance

i) Y3 medium supplemented with 3 M sucrose.
ii) Sterile bottle filled with activated silica gel (2.5 g/embryo)
iii) Sterile cryovials

3.1. Pre growth Procedure

- Incubate sterile embryos (20 nos.) in a conical flask filled with 30 ml of full strength Y3 medium containing 3 M sucrose.
- Place the flask in a rotary shaker for 24 hours (60 rpm).

Desiccation

- ☐ Take out the embryos under aspetic condition from the sucrose medium and wipe it with a sterile tissue paper to remove excess sucrose solution.
- ☐ Place the embryos in sterile bottle filled with activated silica gel (@2.5 g /embryo) for 7 hours.

Thawing, *in virto* retrieval and *ex virto* establishment

Follow same procedure as that for air desiccation method as described in Chapter 2.

3.2. Modified pre growth

Prepare in advance

i. Y3 solid medium containing 0.6 M sucrose
ii. Y3 medium supplemented with 2 M sucrose and 0.25 M Raffinose.
iii. Sterile bottle containing activated silica gel (2.5 g/embryo)
iv. Sterile cryovials

Pre culture Procedure

Incubate sterile empbryos in Y3 (Annexue I) solid medium containing 0.6 M sucrose for 3 days. Incubate sterile embryos (20 nos.) in a conical flask filled with 30 ml of full strength Y3 medium containing 2 M sucrose + 0.25 M raffinose and place in a rotary shaker for 24 hours.

Desiccation

- ☐ Take out the embryos from the sucrose medium and wipe it with a sterile tissue paper to remove excess sucrose solution.
- ☐ Place the embryos in sterile bottle filled with activated silica gel (@ 2.5 g/embryo) for seven hours.

Thawing, *in virto* retrieval and *ex virto* establishment

For thawing keep embroys in water kept at 40 ° C for 2 minutes. Innoculate the thawed embryos into Y3 liquid medium with 60 g/l sucrose for one week and subsequently to 40 g solid Y3 medium. Thereafter follow the same procedure as that for air desiccation method described in Chapter 2.

Various stages of plantlet development in pre-growth desiccation method and nut setting in the field are shown in the following photos.

4. Encapsulation Dehydration

This method is based on the technique developed for the production of synthetic seeds. Here the embryos were embedded in alginate beads so that it can withstand the stresses consequential to desiccation and freezing procedures. The method consists of successive osmotic and evaporative dehydration procedures. Coconut zygotic embryos were suspended in 3 % sodium alginate solution with 2 M glycerine and 0.6 M sucrose dispensed individually into 0.1 M calcium chloride for 20 minutes for the formation of beads. These beads were air desiccated before freezing in liquid nitrogen. Items needed for extraction and surface sterilization is same as that of air desiccation described in Chapter 2.

Items needed for bead preparation

i) Pipette (5 ml)
ii) Pipette dispensor

Prepare in advance

i) Y3 solid medium containing 0.6 M sucrose
ii) 3 % sodium alginate containing 2 M glycerin and 0.6 M Sucrose
iii) Autoclave and keep 0.1 M calcium chloride solution in wide mouthed (100 ml) conical flask
iv) Sterilize the pipette

Pre culture procedure

Pre culture the embryos after surface sterilization in Y3 medium containing 0.6 M sucrose for 3 days

Encapsulation procedure

- Put the surface sterilized coconut embryos in alginate solution
- With the help of a pipette dispenser, the embryo along with alginate solution was sucked and carefully dropped into solution containing 0.1 M calcium chloride for the production of alginate beads embedding the embryo.
- Place it for 20 minutes for the formation of beads.
- Take out the beads from the conical flask and rinse it 2-3 times with sterile water.

Desiccation

- ☐ Place the beads inside the laminar air flow over a sterilized aluminium foil for 26 hours.
- ☐ Put the embryos in sterile cryovials and directly plunge in liquid nitrogen.



***In vitro* retrieval**

- ☐ Inoculate the embryo embedded in alginate beads into Y3 liquid medium containing 60 g/l sucrose and keep for one week.
- ☐ Remove the alginate beads and inoculate the embryos in Y3 medium with 40 g/l sucrose.
- ☐ Follow the culture procedure for ***in vitro*** and ***ex virto*** establishment of plantlet as that for air desiccation method described in Chapter 2.

Different stages of plantlet development of embryos subjected to encapsulation dehydration is shown below.



5. Use of Vitrification Fluids

The solution based vitrification procedures were widely used for the successful cryopreservation of meristems and somatic embryos of various plant species. Among the different vitrification solutions developed by Sakai and his group, Plant Vitrification Solution 3(PVS-3) was found to be very ideal for the cryopreservation of coconut zygotic embryos (Sajini *et al.*, 2010). The embryos is to be precultured in media containing high sucrose that result in mild osmotic shock and protect the cellular membrane that later will help in mitigation of the effect of highly concentrated vitrification solution. The duration of treatment with vitrification solution is very critical for the survival after cryopreservation. In coconut zygotic embryos, it was found to be 16 hours in PVS-3 treatment for reduction of moisture content to 16.8 %. Various steps involved in this method are preculture, dehydration with vitrification solution, unloading, rapid cooling and rapid rewarming. The vitrification protocol developed at CPCRI is detailed in the following.

Items needed for extraction and surface sterilization of embryos is the same as that of air desiccation described in Chapter 2.

Prepare in advance

Y3 medium containing (i) 0.6M sucrose (solid); (ii) 1.2M sucrose (liquid); (iii) 60 g/l sucrose (liquid); and (iv) 40 g/l sucrose (solid)

Pre growth incubation

- ☐ Inoculate surface sterilized coconut embryos into full strength Y3 medium supplemented with 0.6 M sucrose and incubate for 3 days.
- ☐ Place the embryos in plant vitrification solution (PVS-3) containing Y3 medium supplimented with 50 % sucrose and 50 % glycerol in a conical flask (25 embryos in a 100 ml conical flask containing 25 ml PVS-3).
- ☐ Place the conical flask in a rotary shaker (90 rpm) for 16 hours.
- ☐ *Put the embryos in sterile cryovials filled with fresh PVS-3* solution.
- ☐ Plunge the cryovials directly in liquid nitrogen for minimum 24 hours.

Thawing

Take out the vial from the cryotank and immerse the cryovials in warm water (40°C) for two minutes.

Unloading Procedure

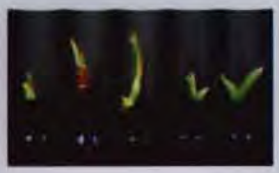
- ☐ Drain off the PVS-3 solution

- ☐ Incubate the embryos in full strength liquid Y3 medium supplemented with 1.2 M sucrose for 90 minutes with one or two changes with the same medium in between.

***In vitro* retrieval**

- ☐ Inoculate the embryos in full strength Y3 liquid medium supplemented with 60 gm/l sucrose.
- ☐ After one month, sub culture into medium (solid) containing 40 gm/l sucrose with BAP and NAA 0.5 mg/l each.

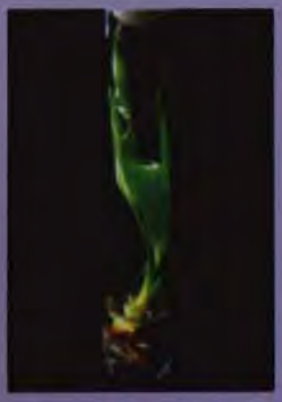
For subsequent sub culturing and *ex vitro* retrieval follow the procedure as that of desiccation method. Different stages of plantlet development of *in vitro* retrieved embryos subjected to PVS-3 are shown below

Validation of the best cryopreservation protocol

Among the four cryopreservation protocols tried, the modified pre growth and PVS3 treatment resulted in more number of healthy plantlets in poly bag within 11-12 months of culturing. The plantlet recovery in poly bag was found to be 22-25% for the afforesaid method.

In order to validate the modified pre growth desiccation method, the embryos from exotic accessions of coconut viz. Fiji Tall and Malayalan Yellow Dwarf were subjected to liquid nitrogen storage. A final recovery of 21% of plantlets in pots in Fiji tall and 28% in MYD were achieved after cryopreservation.




6. Cryopreservation of Coconut Pollen

Pollen Cryopreservation is another technique for long term conservation of genetic diversity. Studies conducted at CPCRI have shown that coconut pollen could be successfully cryopreserved (Karun *et al.*, 2006). Normal nut set has been observed in COD and WCT palms pollinated with cryopreserved pollen from WCT and COD respectively. The factors like germinability and vigour of pollen (pollen tube growth) are to be observed before cryopreserving the pollen.

Checklist for pollen cryopreservation

a) For extraction of spikelets:

i) Pollination bag ii) Sharp knife or scissors
iii) Polythene bag iv) Marking pen

b) For drying of male flowers

i) Petri dish ii) Aluminium foil
iii) B.O.D Incubator iv) Sieve for collecting pollen from the dried male flower

c) For cryopreservation

i) Cryovials ii) Canisters iii) Cryotank with liquid nitrogen.

d) For pollen germination

i) Microslides
ii) Beaker
iii) Measuring cylinder
iv) Cotton
v) Petridish
vi) Germination medium
vii) Microscope (Binocular)

Male flower extraction and pollen collection

In coconut, once in every 20-25 days a fresh inflorescence emerges out from the leaf axil. Male flowers starts opening from the top (basipetal) of each spikelet and sheds pollen continuously for 24 days depending upon the variety and season.

6.1. Procedure for extraction and collection of pollen for storage

i) Bag the inflorescence to prevent contamination from foreign pollen once it completely emerges out of the leaf axil and just before the natural opening of the male flowers starts. Normally it is 6-7 days before the collection of the spikelets.

ii) When the male flowers are about to open from the tip, collect the spikelets in a polythene bag on a bright sunny day between 8-10 AM and label.

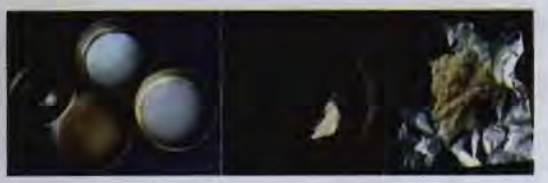
iii) Strip off the male flowers from the spikelets to an aluminium foil placed on a petri dish.

6.2. Dehydration of pollen

i) Keep the petridish with male flowers in an incubator set at 40° C for 24 hours

ii) Extract pollen by sieving the dried male flowers





6.3. Assessement of pollen viability before storage in liquid nitrogen

Preparation of medium for germination of pollen:

i) Dissolve 8% sucrose, 1% agar, 1% gelatin and 0.01% boric acid in distilled water

ii) Boil it until the agar and gelatin gets fully dissolved

iii) Allow it to cool for a while

iv) Take a clean slide and smear the medium uniformly over the slide

v) Allow the medium to solidify

Procedure for pollen germination

i) With help of a small cotton ball disperse the pollen grain evenly over the medium by gently tapping it and holding between the fingers.


ii) Place the slide in a petri dish lined with moist filter paper and cover it to maintain humidity inside the chamber.

iii) Incubate at ambient condition in dark for 90 minutes.

Note: If the ambient temperature is very low (below 25° C), use an incubator with temperature set to 30° C.

Scoring of pollen for germinability

i) Observe the slides under compound microscope.

ii) Score the germinated pollen in 10 randomly selected microscopic fields.

iii) Calculate the germination percentage using the formula

Percentage germination = [No. of germinated pollen in the field / Total number of pollen in the field] x 100

Note: The accepted quality norm for pollen germination was found to be above 25%. The pollen with poor viability may be discarded.

6.4. Cryopreservation procedure

i) Keep the desiccated pollen in the aluminium foil pouch and insert it in to the cryo vial.
ii) Plunge the canister containing cryo vials in liquid nitrogen container.

Retrieval of cryo stored pollen

i) Lift the canisters from the liquid nitrogen
ii) Allow it to thaw for 15 minutes at ambient temperature

Post storage viability assessment: The same procedure may be followed as in the case of desiccated pollen.

6.5. Assessment of nut set after pollination with cryopreserved pollen

It is necessary to test the potential of cryopreserved pollen for seed set under field condition through controlled pollination. Cryopreserved pollen samples were taken out from the cryotank and kept at room temperature for thawing (1 hour). The pollen is subsequently mixed with talcum powder (1: 9) to protect it from heat and becoming too moist.

It was observed that there is no appreciable change in pollen viability after 3 years of storage in liquid nitrogen.

References

Table 1. Per cent pollen germination and vigour (Pollen tube length) µm of WCT and COD pollen at various duration of storage in Liquid nitrogen.

Variety	% Germination	Pollen tube length (µm)
WCT		
Date on cryo storage (13/02/2007)	32.07	221.40
After 1 year	32.16	421.47
2 year	40.05	292.79
3 year	34.32	250.62
COD		
Date on cryo storage (16/02/2007)	46.34	205.60
After 1 year	32.69	213.36
2 year	44.14	213.76
3 year	32.40	224.36

Table.2. Application of pollen Cryopreservation protocol to exotic and indigenous accessions

Name of the Accession	Pollen tube length(µm)		% germination	
	Oven dried	Cryopre-served	Oven dried	Cryopre-served
Exotic				
MYD	295.76	301.14	45.89	44.25
Cochin China	175.45	197.72	26.22	30.47
CRD	322.11	204.27	29.18.	31.61
PHOT	530.23	346.84	27.68	27.24
JVT	284.13	255.25	24.92	21.57
Indigenous				
LCT	304.81	213.23	31.81	30.93
KTOD	246.66	243.19	27.25	29.76
GBGD	237.68	247.68	27.50	25.40
CGD	325.79	293.79	24.20	31.14
AGT	277.39	236.97	25.10	24.65

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Abbreviations

AGT	Andaman Giant Tall
BAP-6	Benzyl aminopurine
CGD	Chowghat Green Dwarf
CPCRI	Central Plantation Crops Research Institute
CRD	Cameron Red Dwarf
GBGD	Gangabondam Green Dwarf
IBA	Indole-3-Butyric Acid
JVT	Java Tall
KTOD	Kenthali Orange Dwarf
LCT	Laccadive Ordinary Tall
M	Molar
MYD	Malayan Yellow Dwarf
NAA	á-Naphthalene Acetic Acid
PHOT	Philippine Ordinary Tall

Annexure I

Y3 Medium

Chemicals	Qty. for 1 litre (mg)	Qty. for 25 l	Stock solution for 25 l	For making 1 l medium
I. MACRO ELEMENTS				
KNO_3	2020	50.50 g	Dissolve the chemicals one by one in 500 ml water, then make up to the final volume of 1000 ml.	Take 40 ml/l from the stock solution
KCl	1492	37.30 g		
NH_4Cl	535	13.375 g		
$NaH_2PO_4H_2O$	312	7.80 g		
II. MICRO ELEMENTS (Fe – EDTA)				
$FeSO_4$	13.9	347.5 mg	Dissolve each chemical separately in 200 ml hot water. Then add $FeSO_4$ to Na_2EDTA with constant shaking. Make up to 500 ml.	Take 20 ml/l from the stock solution
Na_2EDTA	18.5	468.5 mg		
II. a. $CaCl_2$ $2H_2O$				
$CaCl_3$ $2H_2O$	294	7.35 g	Dissolve the chemical in 500 ml water	Take 20 ml/l from the stock solution
II. b. MICRO ELEMENTS				
KI	8.3	207.5 mg	Dissolve the chemicals in 500 ml water	Take 20 ml/l from the stock solution
$MnSO_4$	11.2	280 mg		
$ZnSO_4$	7.2	180 mg		
H_3BO_3	3.1	77.5 mg		
$CoCl_2$	0.24	6.0 mg		
Na_2MoO_4	0.24	6.0 mg		
$CuSO_4$	0.16	4.0 mg		
$NiCl_2$	0.024	0.6 mg		
III. ORGANICS				
Glycine	2.0	50 mg	Dissolve the chemicals in 500 ml water	Take 20 ml/l from the stock solution
Inositol	100.0	2500 mg		
Nicotinic Acid	0.5	12.5 mg		
Pyridoxine	0.5	12.5 mg		
Thiamine HCl	0.1	2.5 mg