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Procedures for Recovering Cassava Clones Distributed *in vitro*

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Contents

In vitro methods have been developed at CIAT for the international exchange of cassava (*Manihot esculenta* Crantz) clones. Sterile cultures in artificial nutritive media are established from disease-free mother plants produced by means of thermotherapy and meristem-tip culture and tested for known cassava viral, bacterial, and fungal pathogens. The cultures for shipment consist of well-rooted plantlets in an agar medium, contained in properly capped 16- × 125-mm test tubes. The test tubes, in turn, are packed within polystyrene boxes.

The tubes are labelled with the clone's name or number; a phytosanitary certificate, issued by the Colombian authorities, and a phytosanitary statement, which provides supplementary information regarding the health status of the cultures, are included in the package.

The *in vitro* system has been accepted by several countries as a means to improve the phytosanitary aspects of the international exchange of cassava germplasm. In the last few years, the technique has been used not only to distribute selected germplasm from CIAT to national programs, but also to introduce into CIAT large numbers of new germplasm collected in the crop's major centers of variability. Furthermore, because of their small size and their disease-free condition and high propagation potential, meristem-derived cultures have been used at CIAT to develop an *in vitro* gene bank of cassava.

The *in vitro* system is only effective as a tool for germplasm exchange if it is managed by well-trained personnel. These guidelines are provided to acquaint the personnel of national programs and other collaborators with the procedures to recover, propagate, and transplant materials from the cultures after arrival at the recipient institution.

The procedures include the following steps:

A. Recovery of the cultures
B. Micropropagation
C. Potting
D. Field transplantation

A. Recovery of the Cultures

It is important to make the necessary arrangements to expedite clearance of the package through customs as soon as it has arrived. Recipients will be advised on the approximate arrival date of the shipment.

Upon their arrival, unpack the test tubes carefully and place them on test tube supports, in an upright position.

1. After short trips (up to 1 week), the plantlets can be potted directly (step C) or can be micropropagated prior to potting (step B) if you have the facilities and personnel.

2. If the shipment has taken 1–2 weeks to arrive at its destination, you

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500 cc.

should expose the cultures to an illumination of 2000 lux (two 40 W fluorescent lamps at 0.50 m above the cultures would be sufficient) with a photoperiod of 14 hours and a temperature of 26–28°C for 1 week. This will allow the plants to recover from the detrimental effects of darkness. After this recovery treatment, the cultures are ready for direct potting (step C) or propagation and potting (step B).

3. After longer trips (more than 3 weeks), and depending on the genotype, etiolation and chlorosis, and sometimes tissue necrosis due to phenolic oxidation, can occur with variable intensity due to the lack of light. You can save extremely deteriorated cultures if any available green bud (terminal or axillary) is aseptically excised and cultured on a fresh medium (step B) immediately upon unpacking. Expose less damaged cultures, especially those with little or no shoot browning, to temperatures of 22–24°C and 1000 lux of illumination for 2 weeks. Even a slight growth of the axillary buds, with or without leaf growth, is sufficient to proceed to the micropropagation step.

B. Micropropagation

Micropropagation is carried out within a laminar flow cabinet or in a transfer room for aseptic work. Make sure to have on hand:

- two No. 10 scalpels, with their handles;
- two forceps: one short (12 cm) and one long (25 cm);
- several petri dishes containing 3–4 sterilized filter papers;
- 2–3 flasks containing sterile distilled water;
- an alcohol burner;
- cotton wetted with 70% alcohol;
- 2–3 flaps (10 × 20 cm each) of sterile filter paper;
- a container with 95% ethanol

The culture medium includes: the mineral salts of Murashige and Skoog + 2% sucrose + 1 mg/l thiamine-HCl + 100 mg/l inositol + 0.05 mg/l benzyl aminopurine + 0.05 mg/l gibberellic acid + 0.02 mg/l naphathalene acetic acid; pH 5.7–5.8; agar = 0.6%. The preparation of this medium is described in Appendix 1.

Equipment and suppliers are listed in Appendix 2.

The procedures for micropropagation are as follows. Steps 3 to 6 are illustrated in Figure 1.

1. Wash hands and arms with soap and running water and wipe with 70% alcohol. If possible, cover the hair with a microbial cap and the mouth and nose with a microbial mask.

2. Immerse the tools in 95% alcohol for 2–3 minutes; flame quickly, and place them under the sterile flaps of paper.

3. Open one petri dish and wet the filter papers with the sterile water taking care to flame the flask. One at a time, uncap 2–3 test tubes (Figure 1-a), and using short forceps, gently pull the entire plantlet from the tube. Place all plantlets within the petri dish (Figure 1-b).

4. Using a scalpel and the short forceps, cut off all expanded leaves and the roots. Then, cut the stem into segments, each one comprising one node (Figure 1-c). Every segment, or "nodal cutting," will then include one axillary bud and a portion of stem (1–2 mm above and 4–5 mm below the bud, respectively). In addition, cut the terminal bud from every shoot, leaving 4–5 mm of stem below the apex. Make sure to make clean, horizontal cuts with the scalpel.

5. Using the larger forceps, gently pick up each "nodal cutting" and each terminal bud, and "plant" each one in an agar-solidified medium (Figure 1-d), in an 18–25 = × 150-mm test tube. Quickly flame the tube and cap it immediately.

During the operation, make sure to frequently wet the tools with 95% alcohol and flame them. Keep the petri dish closed as much as possible.

6. Incubate the "node cutting" cultures at 28°C, under an illumination of 2000 lux until root initiation and, thereafter, under 4000 lux, and maintain a 14-hour photoperiod throughout.

After about 3 weeks, every nodal cutting will give rise to a complete plantlet (Figure 1-e). Once the plantlet has grown to 6–8 cm tall and rooting is proportional to the amount of shoots, the cultures can be subjected to a hardening treatment prior to potting. Hardening is not absolutely necessary, but it will improve the potting.

7. For hardening, increase the illumination to 8,000–10,000 lux and, if possible, decrease the temperature to 24–25°C during 1–2 weeks.

8. Three to four days prior to potting, take the paraffinated paper off the caps, whether or not you have hardened the cultures.

C. Potting

For potting, the following items are needed:

- Pots. Optimal potting is achieved using 3- to 4-inch jiffy-type pots, but clay pots or plastic bags (6.5 × 8 inches) can also be used.
- Substratum, which consists of a mixture of one part soil with three parts of fine sand. The sand must be thoroughly washed with soft water.
- A fair amount of deionized water.
- Long (15–20 cm) forceps.
- Pot labels.
- A water-soluble fertilizer rich in P; e.g., 10–52–10 (N–P–K).
- Plastic, clay, or cement trays with sufficient draining

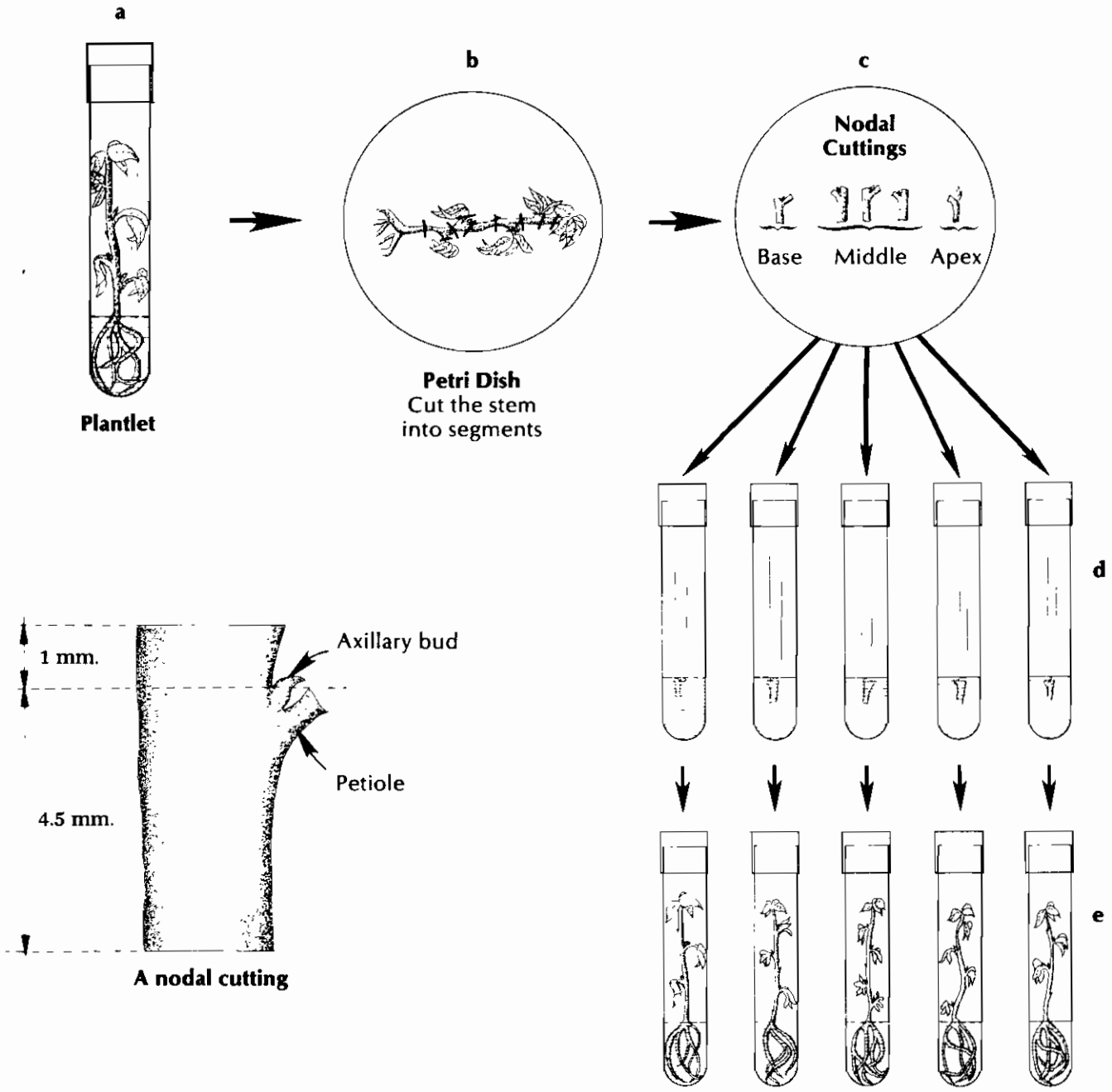


Figure 1. *Micropropagation of cassava through nodal cuttings.*

holes, containing damped sterilized soil.

- A 2.0- × 1.0- × 0.3-m transparent plastic frame to cover the trays.
- A 2.0- × 1.0- × 0.8-m greenhouse table to hold the trays and the plastic frame.
- Two or three tables of similar dimensions to carry out the potting and to hold the pots prior to field transplanting.

You should do the potting in a fresh, but not too cold, location that is protected from direct sun and from insects.

1. A few hours prior to potting, damp with water the soil contained in the trays, place the trays on the greenhouse table and cover them with the plastic frame (Figure 2). A high relative humidity environment will form within the plastic chamber.

2. Sterilize the substratum mixture using steam and fill in the pots. Wet the substratum to about one half water saturation. Make a hole in the center of each pot (Figure 3)

3. Wash your hands thoroughly with soap and water. Uncap the test tubes, one at a time, and, with the aid of the forceps, gently pull out the plantlet (Figure 4). Using the fingers, pull the plant from the tube mouth.

Figure 2. *High humidity chamber includes a plastic frame, trays with damped soil, and a greenhouse table.*

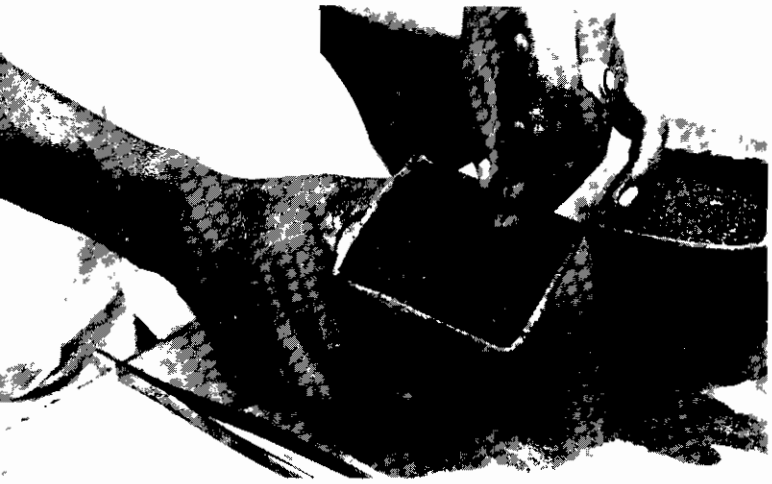


Figure 3. *Making the hole in the pot.*

Figure 4. *Taking the plantlet out of the test tube.*

4. Holding the plant on the palm of your hand, wash off as much agar as possible from the roots (Figure 5), and place the roots and the lower part of the shoot within the hole in the pot (Figure 6). Press the substratum and immediately apply deionized water around the plant (Figure 7).

5. After potting, label the pots and water with the high P fertilizer 10–52–10. Use 2 g fertilizer in 1 liter of water, and apply 80–100 ml solution per pot. Thereafter, when needed and until the time of field transplanting, water with only the high P solution.

6. Place the pots in the high humidity chamber (Figure 8). Five or six days after potting, and thereafter, raise the plastic frame slightly during the fresher hours of the day; close the frame at night. By day 10 or 12, the plastic frame can be taken off completely.

7. After the 12th day, move the pots (still on the trays) to a warmer and much more illuminated part of the greenhouse. In about 2 more weeks, the plants are ready for field transplanting.

The total time taken from receipt to this stage usually is 1 or 2 months depending on whether direct potting or micropropagation and potting was carried out, respectively.

D. Field Transplantation

Successful transplanting can be achieved under cloudy days or during the late afternoon. The soil should be in field capacity and the plants 10–15 cm in height. Use jiffy-type pots or plastic bags and keep root disturbance minimal.

1. Carry the plants in their pots, along with the trays, to the field and cut off the largest leaves.

2. Remove the pots from the tray and place them within the hole large enough to hold the pot and up to the lowest two nodes of the shoot.

3. Press the soil around the plant and water immediately.

4. Maintain high soil humidity for 10–15 days after transplanting, and watch out for ant, worm, or cricket attacks.

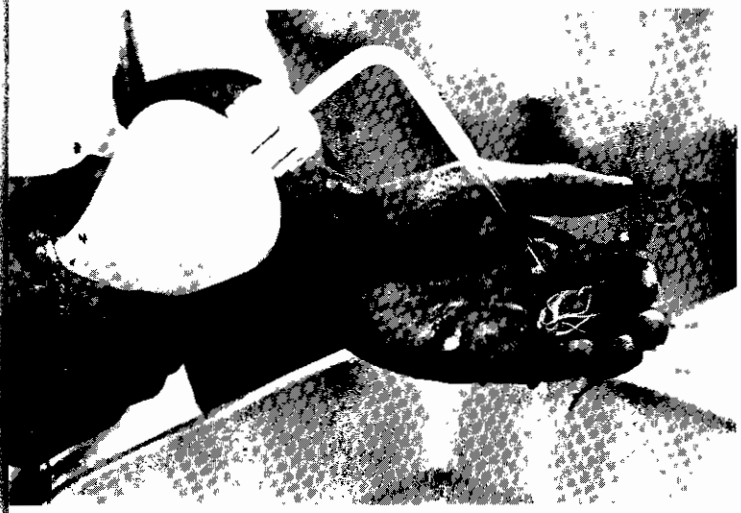


Figure 5. *Washing the roots.*

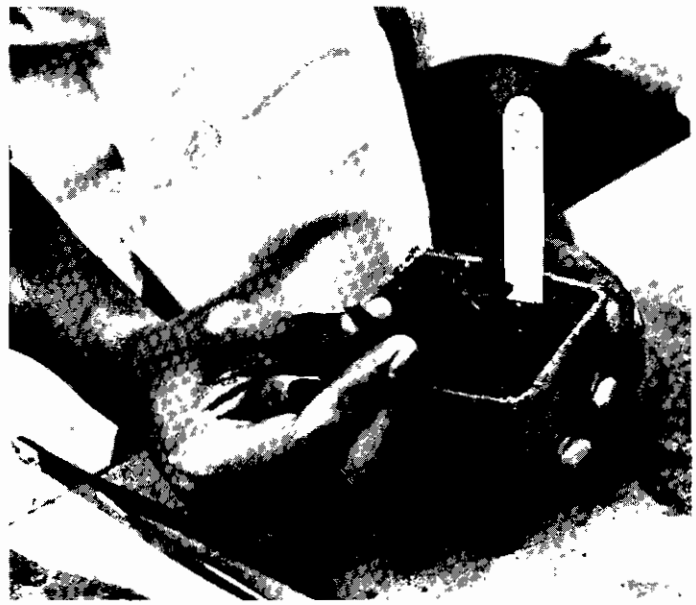


Figure 6. *Potting.*

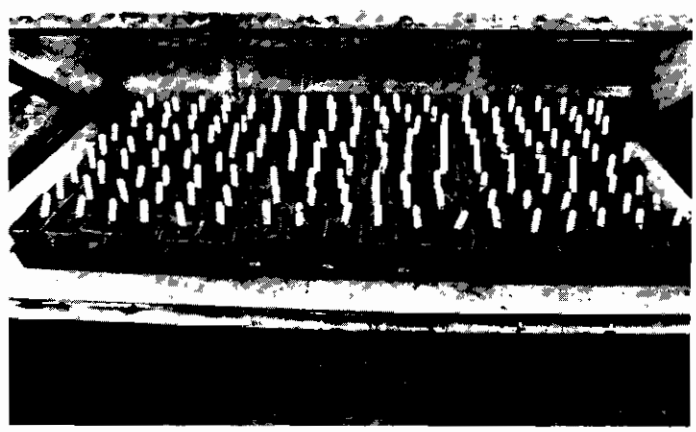
Figure 7. *Watering.*

Figure 8. *Potted plants within the high humidity chamber.*

At the fourth week, the plants should be completely established in the field and can be handled conventionally. However, it is recommended that you pay special attention to protecting the plants from insects or disease throughout the crop. Keep in mind that these plants are elite stocks for the multiplication of clean planting material and that their production, shipment, and handling after arrival have been done with great care and effort.

For news regarding the condition of the cultures upon arrival; any progress developments in handling; or additional information, please write to:

Genetic Resources Unit
CIAT
Apartado Aéreo 6713
Cali, Colombia
S.A.



APPENDIX 1
Preparation of the Culture Medium

A) **Basal Medium.** Can be prepared in either of two forms:

1. Using stock solutions of mineral salts, vitamins, and growth regulators. (For the preparation of the Murashige and Skoog stock solutions, see Appendix 1a).

To prepare 1 liter of medium, to 500 ml of double distilled water add:

20.0 ml of stock solution No. 1
1.0 ml of stock solution No. 2
1.0 ml of stock solution No. 3
2.9 ml of stock solution No. 4
5.0 ml of stock solution No. 5

2. Using the pre-made Murashige and Skoog medium in powder form (without sucrose and without vitamins and agar). Each bag contains 4.3 g of powder, which serves to prepare 1 liter of basal medium. The powder can be stored at 8–10°C, under dessication, for up to 2 years. To prepare the basal medium, dissolve the entire contents of one bag in 500 ml double distilled water. Add the same volumes of stock solutions No. 1 through No. 5 as in 1, above.

B) **Supplements.** Once either of the basal media is ready, proceed as follows:

- Add 5.0 ml of stock solution No. 6 and 6.25 ml of stock solution No. 7
- Dissolve 20.0 g of sucrose
- Add 5.0 ml of the benzyl aminopurine stock solution (see Appendix 1b); 5.0 ml of the gibberellic acid stock solution (see Appendix 1b); 2.0 ml of naphathalene acetic acid stock solution (see Appendix 1b)
- Complete to 700 ml with double distilled water.
- Adjust the pH to 5.7–5.8
- Dissolve by heating 6.0 g of agar in 300 ml of double distilled water.
- Mix well the medium with the agar solution.

C) **Sterilization.** Quickly distribute the prepared medium in 18-X. 150-mm test tubes (5 ml/tubes); let cool slightly and cap the tubes.

Autoclave the tubes with the medium; 15 pounds (121°C) per square inch during 15 minutes; decompress slowly.

Place the tubes in a fresh place until the agar is solid, then store them in darkness at 6–8°C until used.

Appendix 1a. Preparation of Murashige and Skoog Stock Solutions.

To prepare the stock solutions, dissolve, one by one, all the ingredients presented in Table 1, in the volumes of double distilled water shown.

Appendix 1b. Preparation of Growth Regulator Stock Solutions

Benzyl aminopurine (10 ppm): Dissolve 20 mg in a small volume of 1.0 *N* HCl; complete to 200 ml with double distilled water (this is a 100-ppm solution of the hormone): take 20 ml of the 100-ppm solution and complete to 200 ml (this is the 10-ppm stock solution).

Gibberellic acid (10 ppm): Dissolve 22 mg (90% gibberellic acid) in a small volume of 1.0 *N* KOH; complete to 200 ml with water; take 20 ml of this solution and complete to 200 ml with water.

Naphathalene acetic acid (10 ppm): Dissolve 20 mg in a small volume of 1.0 *N* KOH; complete to 200 ml with water; take 20 ml of this solution and complete to 200 ml with water.

Table 1. **Murashige and Skoog stock and medium preparation.**

Stock solution no.[a]	Constituents Substance	Amount	Volume of stock per 1 liter basal medium
1	NH_4NO_3	82.5 g	20.0 ml
	KNO_3	95.0 g	
	$MgSO_4 \cdot 7H_2O$	18.5 g	
	KH_2PO_4	8.5 g	
	Dissolve in 1000 ml water.		
2	H_3BO_3	0.62 g	1.0 ml
	$MnSO_4 \cdot H_2O$	2.176 g	
	$ZnSO_4 \cdot 7H_2O$	0.86 g	
	$Na_2MoO_4 \cdot 2H_2O$	0.025 g	
	$CuSO_4 \cdot 5H_2O$	0.0025 g	
	$CoCl_2 \cdot 6H_2O$	0.0025 g	
	Dissolve in 100 ml water.		
3	KI	0.075 g	1.0 ml
	Dissolve in 100 ml water.		
4	$CaCl_2 \cdot 2H_2O$	15 g	2.9 ml
	Dissolve in 100 ml water.		
5[b]	a) Na_2EDTA	1.492 mg	5.0 ml
	b) $FeSO_4 \cdot 7H_2O$	1.114 mg	
	Dissolve in 200 ml water.		
6	Thiamine-HCl	10 mg	5.0 ml
	Dissolve in 200 ml water.		
7	*m*-inositol	0.8 g	6.25 ml
	Dissolve in 200 ml water.		

a. Stocks 2 and 6 should be kept frozen; all the others at 8–10°C. Keep stock 5 protected from light.

b. Separately dissolve a and *b* in 50 ml water each; heat up *b* in a water bath; mix both solutions well; let cool and then add water to complete to 200 ml.

Addition of Growth Regulator Stocks to the Medium: To determine the volume (Appendix 1) of each growth regulator stock solution necessary to obtain the prescribed concentrations (step B), apply the following formulation:

$C_1V_1 = C_2V_2$

C_1 = Concentration of stock = 10 mg/l

C_2 = Final concentration of growth regulator in the medium:
Benzyl aminopurine = 0.05 mg/l
Gibberellic acid = 0.05 mg/l
Naphathalene acetic acid = 0.02 mg/l

V_1 = Volume (in ml) of stock solutions needed = X

V_2 = Final volume of medium = 1000 ml

$$X = \frac{0.05 \text{ mg/l} \times 1000 \text{ ml}}{10 \text{ mg/l}} = 5.0 \text{ ml of either benzyl aminopurine or gibberellic acid.}$$

$$X = \frac{0.02 \text{ mg/l} \times 1000 \text{ ml}}{10 \text{ mg/l}} = 2.0 \text{ ml of naphathalene acetic acid}$$

APPENDIX 2.
List of Suppliers

A) Equipment

1. Laminar air-flow cabinets[a]
   — Environmental Air Control (TT3630 = Table Top)
   747 Bowman Ave.
   Hagerstown, Maryland 21740
   U.S.A.
   — Viro-mart Ltd.
   Box 182
   Burlington, Ontario
   Canada L7R 3Y2

2. Portable sterilizer, pressure cookers
   — Fisher Scientific Co.
   1815 E. Commercial Blvd.
   Ft. Lauderdale, Florida 33308
   U.S.A.
   — American Scientific Products
   1900 N.W. 97th Avenue
   P. O. Box 520276
   Miami, Florida 33152
   U.S.A.

3. Balances, analytical (0.1 mg readability)
   — American Scientific Products

4. Hot plate/stirrer
   — Fisher Scientific Co.

5. pH meters
   — Fisher Scientific Co.
   — American Scientific Products
   — Cole Parmer Instruments
   7425 North Oak Park Ave.
   Chicago, Ill. 60649
   U.S.A.

B) Glassware

1. Culture tubes (Pyrex or Kimax brand): 18–25 × 150 mm
   — Fisher Scientific Co.
   — American Scientific Products

2. Culture tubes caps (Kim-CAP closures, autoclavable, natural color, O.D. 18–25 mm)
   — Arthur H. Thomas Co.
   P. O. Box 779
   Philadelphia, PA 19105
   U.S.A.

3. Petri Dishes (Pyrex brand): 15 × 100 mm
   — Fisher Scientific Co.
   — American Scientific Products

4. Beakers (400, 600, and 1000 ml) and Erlenmeyer flasks (250 ml) (Pyrex brand):
   — Fisher Scientific Co.
   — American Scientific Products

5 Pippets, serological (2 in 1/10 ml, 5 ml, 10 ml)
   — Fisher Scientific Co.
   — American Scientific Products

6 Graduated cylinders (50, 100, 250 ml)
   — Fisher Scientific Co.
   — American Scientific Products

C) Surgical Tools

1. Dissecting blades, size No. 10 and No. 11

2. Knife handles for blades No. 10 and No. 11

3. Forceps (normal) and extra-long forceps
   — Fisher Scientific Co.

D) Chemicals

1. Six major mineral salts for Murashige and Skoog medium
   — Fisher Scientific Co.
   — American Scientific Products

2. Seven minor mineral salts for Murashige and Skoog medium
   — Fisher Scientific Co.
   — American Scientific Products

3. Pre-made Murashige and Skoog salt mix: Catalog No. 500-1117, without vitamins and without sucrose and agar:
   — Gibco Labs.
   3175 Staley Road
   Grand Island, New York 14072
   U.S.A.

4. Thiamine-HCl, *m*-inositol
   — Sigma Chemical Co.
   P. O. Box 14508
   St. Louis, MO 63178
   U.S.A.

5. Growth regulators: benzyl aminopurine, gibberellic acid, naphathalene acetic acid:
   — Sigma Chemical Co.

6. Sucrose[b]
   — Sigma Chemical Co.

7. Agar (Difco Bacto Agar)[c]
   — Difco Laboratories
   Detroit, Michigan
   U.S.A.

E) Others

1. High P fertilizer ("Plant Prod" 10–52–10)
   — Plant Products Co. Ltd.
   314 Orenda Road
   Bramalea, Ontario L6T 1G1
   Canada

2. Jiffy pots (3–4 inch)
   — Jiffy Products (N.B.) Ltd.
   Shippengam
   Canada

3. Culture tube racks (45° slant)
   — Limited Plastic
   P.O. Box 89

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a. Can be built as long as proper filter is identified. One HEPA filter (24 × 48 inches) costs about US$200.00. Additionally, a centrifugal 1/3 H.P. blower (1800–2000 r.p.m.) will be needed. The air prechamber and the base can be made of wood and the walls of acrylic.
b. Can be replaced by commercial crystalized table sugar.
c. Can be replaced by agar ribbons, provided they are thoroughly washed overnight.

Lemoncove, California 93244
U.S.A.

4. Plastic (autoclavable) caps (25 mm D.), trays, vessels, etc.
— Magenta Corporation
4149 W. Montrose Ave.
Chicago, Illinois 60641
U.S.A.

Complementary References

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CIAT is a nonprofit organization devoted to the agricultural and economic development of the lowland tropics. The government of Colombia provides support as host country for CIAT and furnishes a 522-hectare site near Cali for CIAT's headquarters. In addition, the Colombian Foundation for Higher Education (FES) makes available to CIAT a 184-hectare substation in Quilichao and a 73-hectare substation near Popayán; the Colombian Rice Federation (FEDEARROZ) also makes available to CIAT a 30-hectare farm—Santa Rosa substation—near Villavicencio. CIAT also co-manages with the Colombian Agricultural Institute (ICA) the 22,000-hectare Carimagua Research Center in the Colombian Eastern Plains and carries out collaborative work on several other ICA experimental stations in Colombia; similar work is done with national agricultural agencies in other Latin American countries. CIAT is financed by a number of donors represented in the Consultative Group for International Agricultural Research (CGIAR). During 1994 these CIAT donors are the governments of Australia, Belgium, Canada, France, the Federal Republic of Germany, Italy, Japan, the Netherlands, Norway, Spain, Sweden, Switzerland, the United Kingdom, and the United States; of America; the European Economic Community (EEC); the Ford Foundation; the German Agency for Technical Cooperation (GTZ); the Inter-American Development Bank (IDB); the International Development Research Centre (IDRC), the International Fund for Agricultural Development (IFAD); the OPEC Fund for International Development; the Rockefeller Foundation; the United Nations Development Programme (UNDP); the United Nations Food and Agriculture Organization (FAO); the World Bank; and the W. K. Kellog Foundation.

Information and conclusions reported herein do not necessarily reflect the position of any of the aforementioned entities.