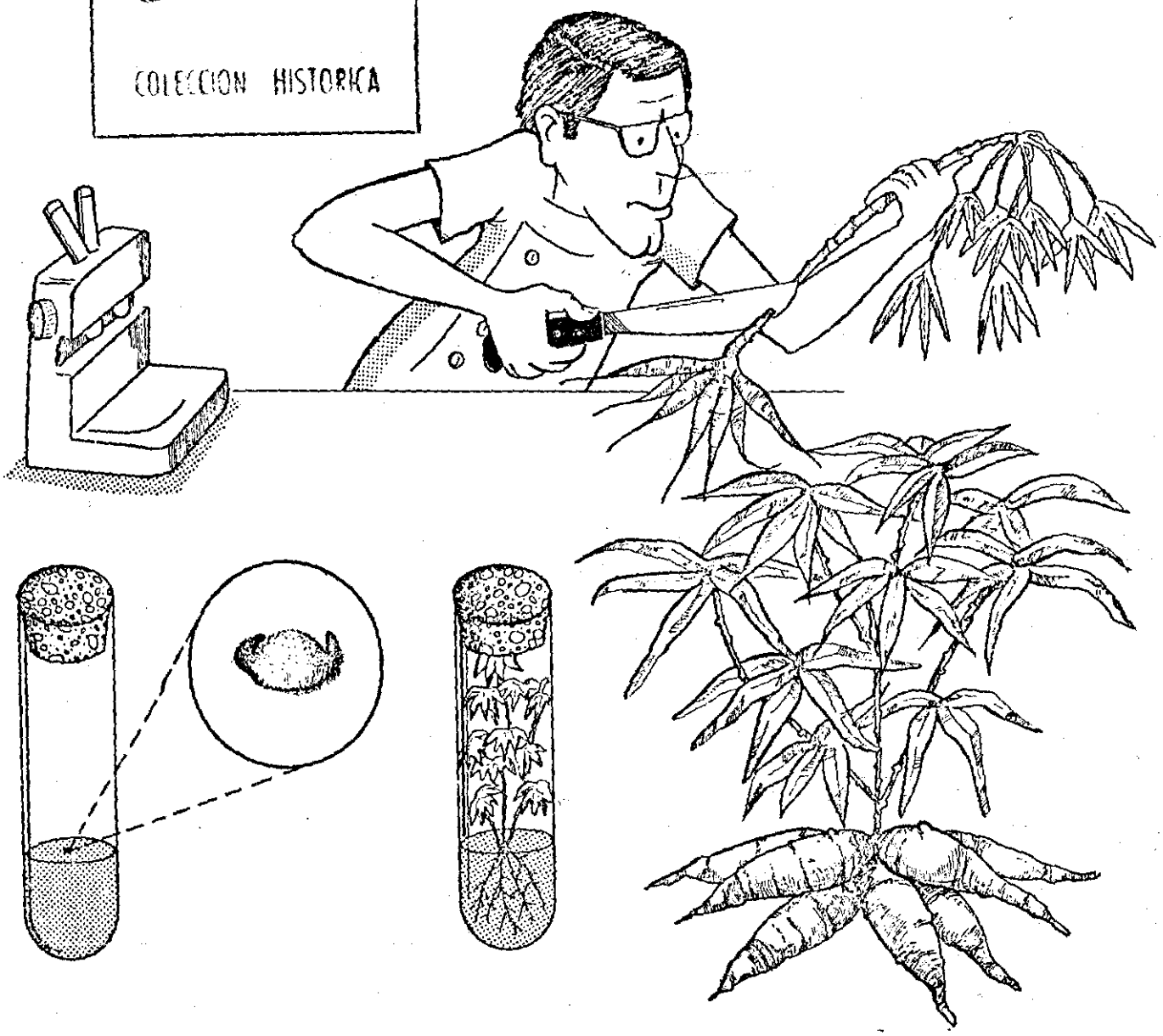

MERISTEM CULTURE IN CASSAVA

principles and methodology

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MERISTEM CULTURE IN CASSAVA

Principles and Procedures

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P R E F A C E

Recent advances in the methods of plant tissue culture have made it possible to use these techniques as tools for the study of both basic and applied problems in agriculture, horticulture, forestry and industry.

It is now possible to cultivate plant cells, to induce callus to form organs and to grow whole plants from apical meristems. Certian methods can be used to vary ploidy levels in cells and plants; even protoplasts can be induced to produce callus and plants.

However, with the exception of meristem and shoot-tip culture methods, most of these techniques have not developed yet to the stage that could be applied on a wide scale.

The propagation of economically important crop plants by means of meristem culture provides an efficient means to free the plants from systemic pathogens; furthermore due to their inherent genetic stability, meristem cultures can be utilized to build up gene banks. These applications are particularly valuable in vegetatively propagated crops like cassava.

The final objective of this work is to provide information, as complete as possible, on the principles and methodologies of meristem culture in cassava. In doing so, care was taken to provide both general background information on every aspect of the technique and detailed step by step procedures drawn from our research. Indeed, various of the techniques here presented could, after proper adjustment, be applied to other crops as well.

The most immediate goal of this preliminary work is to expose its contents, especially the technical protocols to the critical review by those who will use it on the first instance and by others who may go through it. The suggestions received will be used to make both a solid conceptual document and, above all, a workable handbook.

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W. M. Roca

SECTION A

MERISTEM CULTURE IN CASSAVA: GENERAL METHODOLOGY

I. SOURCE OF PLANT MATERIAL

1. Select 10-12 cm stem cuttings containing 3-5 dormant buds.

2. Desinfest the cuttings superficially: Dip the stakes for 5 minutes in a solution made of 6 g. of each Bavistin and Orthocide per one liter of water; then let the cuttings dry for 1-2 hours (1).

3. Seal the upper cut ends with melted parafin.

4. Plant the cuttings in pots containing sterilized soil.

5. Water the pots with 1/3 strength Hoagland's nutrient solution (see Table 1) or with a 15-15-15 soluble fertilizer-diluted to 1/5.

6. Place the pots in the greenhouse or growth chamber at relatively low light intensity and an average temperature of 28°C.

II. PREPARATION OF STERILE TISSUE

The cuttings planted as above will provide a convenient, and relatively continuous, supply of buds for the excision of apical meristems.

In about two weeks the buds have sprouted, and some leaves have expanded; remove the terminal bud together with 2-3 cm shoot axis.

TABLE 1. HOAGLAND's NUTRIENT SOLUTION

Stock No.	Constituents	Composition of Stock* Solutions (g)	Volume (ml) of Stocks for One Liter Nutrient Sol. Full	1/3	1/5
1	$Ca(NO_3)_2 \cdot 4H_2O$	23.6	5.0	1.6	1.0
2	KNO_3	10.2	5.0	1.6	1.0
3	$MgSO_4 \cdot 7H_2O$	24.6	2.0	0.6	0.4
4	$NH_4H_2PO_4$	12.0	1.0	0.3	0.2
5	H_3BO_3	2.8	1.0	0.3	0.2
	$MnSO_4 \cdot H_2O$	3.4			
	$CuSO_4 \cdot 5H_2O$	0.1			
	$ZnSO_4 \cdot 7H_2O$	0.2			
	$(NH_4)6Mo7O_{24} \cdot 4H_2O$	0.1			
	H_2SO_4 conc.	0.5 ml			
6	Na_2EDTA	1.492	5.0	1.6	1.0
	$FeSO_4 \cdot 7H_2O$	1.114			

---

* Dissolve constituents as follows: Stock 1 in 100 ml water; Stock 2 in 100 ml; Stock 3 in 100 ml; Stock 4 in 100 ml; Stock 5 in 100 ml and Stock 6 in 200 ml.

Under greenhouse conditions (20-30°C) one can collect 3-5 buds per stake every 15 days. Under thermotherapy temperature (35-40°C), the collection interval may be shortened to about one week.

Actively growing vegetative buds, but not flowering, are most convenient as a source of meristems.

Apical meristems are generally sterile in their natural state, even though the outer appendages of the bud may carry contaminating micro-organisms. Meristems excised from materials grown in clean greenhouse or growth chamber maintain their sterility without surface desinfestation; nevertheless, the buds need to be thouroughly rinsed with sterile destilled water and the dissecting tools sterilized during the surgical operations.

The following protocol can be followed to surface desinfest the buds prior to dissection:

1. Quickly rinse the buds in 70% ethanol.

2. Soak the buds for 5-6 minutes in a solution of calcium hypochlorite prepared as follows: dissolve 5 g. in one liter of water, filter twice and dilute 1:4 with water.

3. Wash the buds 4-5 times with sterile destilled water.

III. ISOLATION OF APICAL MERISTEMS AND SHOOT TIPS

Herein, the term "apical meristem" is refered to a 0.4-0.5 mm structure, comprising the dome shaped meristem tip flanked by one or two of the youngest primordial leaves. The term "shoot tip" describes a larger structure, comprised by

the apical meristem plus several leaf primordia and a portion of the sub-apical axial tissue.

a. Dissecting tools:

1. A stereomicroscope with a lamp.
2. Two dissecting knives No. 11.
3. Two forceps.
4. Two micro-scalpes made of razor blade fragments, with a single cutting edge of 5-7 mm., cemented to a small wooden stick.
5. Two fine bent needles of 3-5 mm., made of No. 25 needles.

b. Sterilization of dissecting tools:

Flame sterilization of dissecting tools may produce loss of temper and oxidation of the metal. Also, carbonized organic matter, adhered to the tool, is difficult to remove without causing damage.

The following protocol should be followed as frequently as possible:

1. Immerse knives, forceps, needles, scalpes in 70% ethanol for 1-2 minutes.
2. Let air dry or dry with sterile filter paper.

If the aim is the elimination of systemic viruses, the sterilization must begin by dipping the tools in saturated soapy water and then follow steps 1-2.

c. Excision techniques:

Air borne contamination of the cultures can be almost eliminated with the use of a laminar flow cabinet during the surgical operation. Otherwise, the laboratory or room should be dust wiped, the windows sealed so as to avoid air movement and the bench and walls washed with an antiseptic prior to the operation. Within a working session, the bench should be frequently washed with 70% ethanol. If bactericidal ultra violet lamps are installed, these should be turned on at least 30 minutes before commencing the work; and off just before entering into the room.

Satisfactory volumes of high-quality work can be expected if attention is paid to: maintaining the sterility, preventing the desiccation and avoiding mechanical injury of internal succulent structures of the bud. Commonly, injury of the apical meristem may result in the gelation of the cytoplasm which is recognizable by a change of the tissue appearance to a whitish translucent. Also, such injury may result in loss of turgor and cellular collapse (2).

For further information on general care and organization of the laboratory see the Chapter 2 of H. E. Street's Book (3).

d. Operation:

1. With a pair of forceps hold with one hand the desinfested bud, over the microscope stage. The stage has been previously wiped with 70% ethanol and let dry.
2. Under a magnification of 10-15 x, work inward

removing the outer appendages (leaves and stipules) with a dissecting knife. Larger appendages will snap off readily when bent away from the axis.

3. As you progress into the inner structures, these become pale green in appearance. Still enough leaves remain to protect the apical meristem. At this stage of dissection, the apex comprises 4-5 primordia and measures 0.8-1.5 mm; this is called the "shoot tip." A shoot tip can be readily excised for culturing by a cut along its morphological neck. The shoot tip will adhere to the surface of the knife for transfer to the agar medium.

4. If the culturing is aimed at eradication of systemic viruses, the surgical operation continues inwards. Adjust the position of the apex, and focus the microscope so that the tip of the apex can be seen in profile.

5. Dissect out the leaf primordia, by cuts along their bases in order to prevent injuring the apical meristem.

6. Increase the magnification of the microscope (25-30x). Now, the youngest primordia can be seen on the flanks and partially enclosing the meristem.

7. With a curved needle, snap off these primordia, taking care not to injure the apical meristem.

8. Cut away excess of leaf bases and axial tissues.

9. The 0.4-0.5 mm apical meristem has a dome-shaped, brilliant, tip and comprises 1-2 radially simmetric primordia.

10. Using the microscalpel, the apical meristem can be finally excised. Tilt the apex so that it can be seen in profile and make the final cut on a downward

pass. Some workers prefer to place the apex vertically and to make two successive oblique cuts, one across the other and along the flanks of the meristem.

11. The apical meristem will adhere to the tip of the scalpel and thus can be transfered quickly into the test tube and planted on the agar medium.

The various steps in the surgical operations, especially the final ones, should be handled as rapid as possible or the tissue may get damaged due to dehydration or cementing to the scalpel.

IV. INCUBATION OF CULTURES

1. Meristems planted on the surface of agar media, in test tubes, are placed in racks and incubated in a growth chamber or in a temperature and light controlled room.

It is desirable, but not absolutely necessary, to place the meristem explant in an upright position on the agar. The meristem grows somewhat faster and roots more easily when placed upright.

2. During the initial stages of growth, the incubation room is maintained under the following conditions:

Day temperature:	28 $\pm$ 2°C
Night temperature:	24 $\pm$ 2°C
Illumination:	3000-4000 lux
Photoperiod:	16 hours
Light quality:	Flourescent lamps, tropical day light type.
Air humidity:	Should be maintained high enough to minimize moisture loss from the culture media.

3. Prepare the differentiated plantlets for potting.

V. HARDENING OF CULTURES

Often, the technique of meristem propagation is upset due to the failure of the transfer step from the test tube to pots. A successful tissue culture method should result in high frequency re-establishment in soil of the tissue culture derived plants.

We found that potting is greatly expidited if 2-3 cm test tube plantlets are subjected to the following prior treatment:

1. Increase the illumination to about 15,000 lux.

2. Reduce the temperature to 25°C day and night.

3. Reduce the air humidity inside the culture vessel, this is accomplished by loosening the test tube caps.

VI. POTTING

1. Transfer the hardened cultures to the greenhouse to carry out the potting operation.

2. As substratum, use two parts of vermiculite and one part of fine sand. This mixture provides adequate drainage and aeration.

3. Fill up the pots (we use "jiffy" pots) with the substratum and wet slightly with low salt water. Jiffy pots offer the advantage that they can be directly transplanted to the field with no disturbance to the roots.

4. Uncap the test tube, pull the plantlet from the agar and

thoroughly was the roots with water.

5. With a wooden stick make a hole in the center of the substratum.

6. Insert the roots and a portion of the stem into the hole and press the substratum into firm, but not too hard, contact with the roots.

7. Water the pots generously, label and place them in a high relative humidity environment. This is readily achieved in a plastic covered cage placed on the top of a greenhouse table; within the cage, the pots are placed on flats containing wetted sterile soil. This system resulted more efficient than the use of an inverted glass beaker to cover each plant or the use of intermitent mist as described elsewhere (4, 5).

   Conditions recorded in the cage are: day temperature around 40°C; night temperature: 20°C; the relative humidity during the night reaches up to 100% and during the day, around 85%; the light intensity varies around 30,000 lux.

8. Try not to water the plants directly. Apply the water to the trays; the water will penetrate the pots by capillatiry.

   We have recently found that plantlets watered once, at the time of potting, with a high phosphorous soluble fertilizer took on faster and grew more vigorously than the plants watered with Hoagland's nutrient solution.

VII. TRANSPLANTING TO THE FIELD

1. Prior to field transplanting, the plants are exposed to lower air humidity by opening the lid of the cage gradually.

2. After 3-4 day acclimatation, the plants are ready for transplantation. A plant of 10-15 cm, comprising about 10 expanded leaves, seems to be at the right stage for field transplanting.

3. Choose a cloudy day to carry out the transplant; otherwise make it late in the afternoon.

4. The soil should be adequately humid.

5. Do not move the pots from the flats; carry them together to the field. Thus, the roots will not be disrupted.

6. Open a hole in the soil, large enought to hold a pot.

7. Insert the pot into the hole, press the soil firmly around the pot and a portion of the stem as well.

8. Cut off the largest leaves from each plant in order to minimize evapotranspiration.

9. Water the plants with a starter fertilizer, if possible.

SECTION B

MERISTEM CULTURE: GENERAL RESPONSES

The apical meristem is a small (0.2 - 0.3 mm) dome of tissue located at the pinnacle of the shoot. The apical meristem is first formed during embryo development and, except for periods of dormancy, remains active throughout the life of the plant. The apical meristem not only constitutes a self-perpetuating structure, but is also responsible for the continuous, indeterminate, formation of tissues and organs of the shoot. The vegetative perpetuation of cassava is due to the activity of the apical meristems localized in the axillary buds of the stem.

While the meristem gives rise to the tissues of the plant body, these in turn produce the nutrients required for the meristems. It has been shown that an isolated apical meristem is capable to regenerate an entire plant (6) as long as a proper chemical and physical environment is provided *in vitro*. The totipotency of the apical meristem forms the basis for the technique of meristem culture.

In practice, the size of the apical meristem used as explant influences the rate of plant regeneration. Thus, we have confirmed reports (7) that in cassava, very small explants (less than 0.2 - 0.3 mm) tend to form a callus without organogenesis. However, plants can be regenerated from very small apical domes if a more appropriate environment for culture is devised; in fact, it seems now possible to regenerate plants even from cassava mesophyll protoplasts (see Section I).

I. SEQUENTIAL DEVELOPMENT OF THE CULTURES

The standard development of a cassava meristem explant, isolated and cultured as described herein, into an entire field-grown plant can be divided into various stages; these comprise both laboratory and greenhouse phases (see Fig. 1).

1. During the first week of culture, little morphological differentiation can be observed if the meristem explant is 0.4 - 0.5 mm. During this incubation stage, a slight increase in tissue volume can be noted. The development of some pale-green pigmentation may be evident, being this an early indication of culture survival. If, on the contrary, the explant becomes white translucent this may be an indication of injury during excision. Larger explants (i.e. shoot tips), double their fresh weight in 4-6 days, as well as begin to differentiate right away; leaves grow at the tip of an elongated stalk.

2. During the next 2-3 weeks, the main events leading to the differentiation of the explants into organized shoots and roots take place.

   Following the increase in tissue mass, the upper part of the explant becomes greener (the shoot) and the lower part remains whitish or yellowish (the root pole).

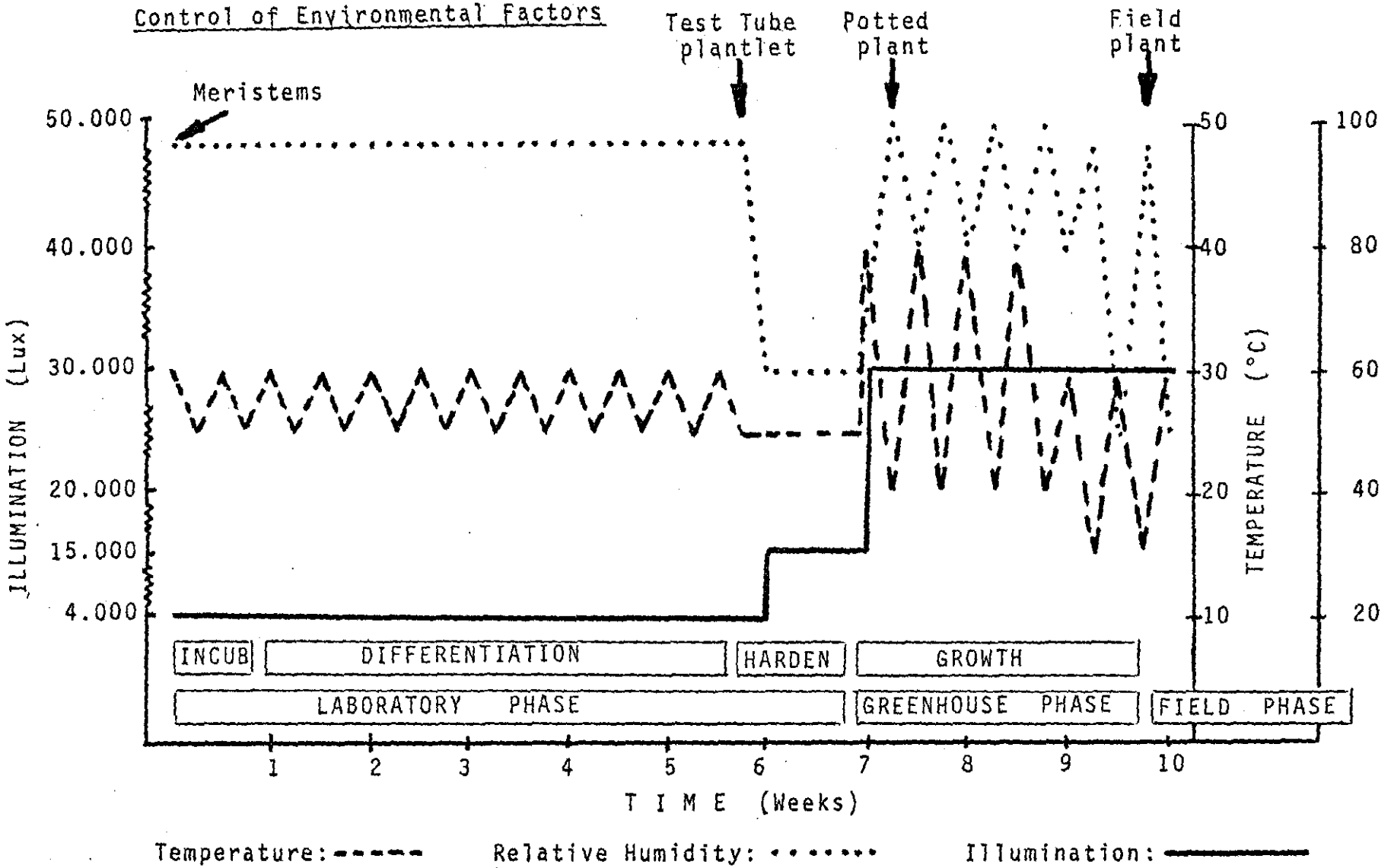
   By the third-fourth week, a shoot stalk has been formed and a few leaves have begun to expand. Very often, the formation of a callus precedes the growth of roots.

3. By the fourth-fifth week, a 2-3 cm rooted plantlet has developed.

   The general pattern of development may suffer certain

FIG. 1. DEVELOPMENTAL STAGES OF CASSAVA MERISTEM CULTURES:

Control of Environmental Factors



alterations depending on the cassava variety, the composition of the culture media as well as the interactions of both:

a. A shoot forms without roots; very often a wound callus grows at the base of the shoot and sometimes the roots will begin to grow after a long period of culture.

b. A small shoot, with very long roots, is formed.

c. The shoot remains very small without rooting; this is often accompanied by a deposition of yellowish-brown exudates in the agar which probably are inhibitory to the growth of the meristem.

It seems that the ability of the meristems to root in vitro is the main difference among cassava varieties.

Shoot differentiation can be achieved readily. The lack of rooting behaviour of a variety can be overcome by altering the composition of the culture medium. However, from the practical stand point, this approach would not be too workable, especially if one has to handle hundreds or even thousands of different varieties. Instead, we have devised a simple technique to produce rooted plantlets very quickly:

-- The culture media is formulated (see Section C), so as to enhance quick shoot differentiation, regardless of rooting.

-- After 3-4 weeks of culture, shoots have formed; then 0.5 - 1.0 cm stem tips are cut from the shoots and planted aseptically on media (see Section C) to promote rooting and further shoot growth.

-- In about one week, 2-3 cm shoots, with root initials and without callus, have been formed. Thus, this technique allowed us, not only to produce plantlets from any cassava variety so far tested, but also to avoid the formation of callus at the shoot-root transition zone.

4. At about the fifth week, from the initiation of the culture, the differentiated plantlets will be subjected to a hardening treatment prior to potting (see Fig. 1).

The hardening stage imparts to the plants some tolerance to moisture stress and thus, facilitates the conversion of the plants from a heterotrophic (in vitro) to an autotrophic state (in pots) (8), with typical terrestrial water control.

It has been found that water loss at transplanting of meristem derived plantlets was related to reduced quantities of epicuticular wax during culture and to incomplete vascular development between roots and shoots, thus restricting acropetal water transport (9).

We have achieved near 100% plant survival at potting through the application of the following treatments to the meristem derived cassava cultures:

a. The preparation of the cultures actually begins at the stem tip cutting step, whereby roots initiate directly at the base of the cutting without the interference of a callus. The lack of basal callus probably favors a direct connection of the root and shoot vascular systems.

b. At about the fifth week of culture, the rooted stem

tips are subjected to 25°C day and night temperature regime and to higher (15,000 lux) illumination during about two weeks. These conditions probably promote the formation of harder leaf lamina as well as favor the initiation and growth of roots. In fact, varieties that did not root at the higher, culture room temperature, did so at 25°C.

c. Three-four days prior to potting, the humidity of the culture is reduced by loosening the test tube caps. The reduction in culture humidity probably enhances the development of epicuticular wax (9) on the leave.

d. At about the eighth week, the 4-5 cm plantlets are ready for potting.

e. The substratum used, the high phosphorous fertilizer applied and the high humidity (80-100%) cage to keep the potted plants, all contribute to a quick establishment of the plants. The temperature in the cage fluctuates widely so as to mimic field conditions (see Fig. 1). The light intensity is raised to an average of 30,000 lux.

f. After two-three weeks, the plants are gradually exposed to ambient conditions and then moved to the field for transplanting.

II. MEASUREMENT OF GROWTH AND DATA RECORDS

The initial stages in the development of meristem cultures are difficult to measure, however a description of developmental events can always be made. In fact, various tissue culture systems may only be accesible to visual observation, both macroscopic and microscopic.

Some of the developmental processes can easily be quantified. For example, percentage of cultures forming shoots, roots or shoots and roots simultaneously. Also, if the nature of the experiment so requires, determinations can be made of fresh weight, internodal elongation, etc. Even the number of cells/unit fresh weight or dry weight, formed within a period of culture, can be determined; maceration and cell counting methods are available.

However, from a practical standpoint, we need to know: propagation rates of cultures, i.e. number of sub-cultures and number of regenerating shoots per sub-culture. Also important, is the percent of viable cultures and the percent of disease-free plants produced.

Statistical analyses of the results should be carried out whenever possible; analysis of variance, treatment means and L.S.D. values of an experiment should be calculated (10).

Often we wish to find out the response of meristems to one or more constituents of the medium. Fig. 2A shows an experimental design to determine the effect of a wide range of concentrations of BAP and the synergetic response with GA. Fig. 2B (10) graphically depicts the sub-threshold, threshold, sub-optimal, optimal, supra-optimal and inhibiting responses of the culture, to a range of concentration, of a media constituent. These data will provide valuable information for the adjustment of media components to achieve specific objectives.

FIG. 2

A. An experimental design to test the effect of various concentrations and combinations of GA and BAP.

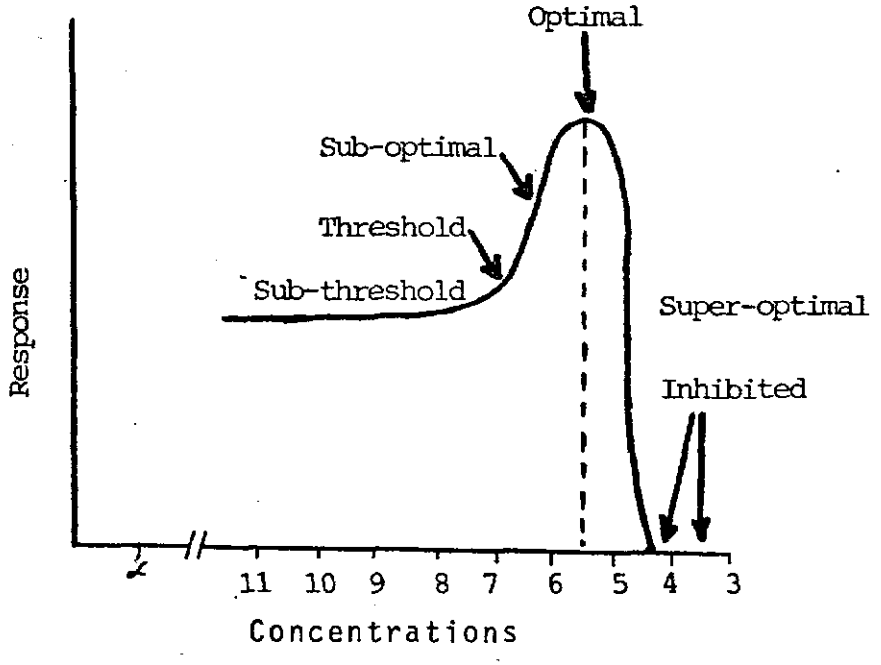
B. An ideal curve response of tissue cultures to various concentrations of a constituent of the culture medium. (From De Fossard, R.A. 1976) (10).

A.

B A P mg/l

GA mg/l	0	0.02	0.04
0	1	2	3
0.05	4	2	6
0.1	7	8	9

B.



SECTION C

MERISTEM CULTURE MEDIA

The composition of the culture medium is one of the most important factors in the successful culture of apical meristems. The culture medium should provide the nutrients and growth factors necessary to the differentiation of an isolated apical meristem into a complete plant. Along with the chemical nutrients of the culture medium, the physical milieu (i.e. medium consistency, oxigenation, light, temperature, etc.) is also important.

I. FORMULATION OF GENERAL STOCK MEDIA FOR MERISTEMS CULTURE.

In general, the media for the culture of apical meristems contain the following ingredients:

1. Inorganic nutrients
2. A source of carbohydrate
3. Vitamins
4. Organic supplements (rarely used)
5. Hormonal supplements

The inorganic nutrients are salts which supply the need for all macro-elements and micro-elements. Adequate concentrations of these elements have been prescribed (11,12): at least 25 mM each of nitrate and potassium, excess of 8 mM in ammonium may be detrimental, 1-3 mM of calcium, sulfate and magnesium may be enough; the micro-nutrients should include I, B, Mn, Zn, Mo, Cu, Co, and Fe. Iron is normally added as chelate to ensure its avail

ability over a wide range of pH (13).

As carbohydrate, 2-4% sucrose is widely used.

The vitamins thiamine, pyridoxine and nicotinic acid are commonly used; however, thiamine may be the only one required (12).

The macro and micro mineral nutrients plus the sucrose and the vitamins constitute the basal medium.

There are at least ten formulations of basal medium which have been named after their author(s); e. g. Murashige-Skoog, Gamborg, et al, Schenk and Hildebrant, Nitsch, etc. (13). The medium of Murashige-Skoog (14) has been used as a basal medium for the culture of apical meristems of a wide range of species (8). This medium, supplemented with the vitamins of Gamborg's et al medium was used for cassava (7) with success. For the culture of cassava apical meristems we routinely use as basal medium the Murashige-Skoog mineral salts and vitamins with a higher level (10 mg/1) of thiamine. The mineral salts of Murashige- Skoog are now available in a premade powder form from "Gibco" (14a).

Certain organic supplements (coconut milk and other plant extracts, etc.) have been added to the basal medium. Casein hydrolysate has been used as a source of amino acids. These suplements, however, have proved unnecessary in most meristem cultures and their use is not encouraged due to their undefined chemical composition.

Cassava apical meristems can be kept alive, though for a limited period of time, in a minimal basal medium. In order to maintain the meristematic potential during growth and development of the cultures, it is necessary to suplement the medium with an adequate balance of hormones. The class and concentra-

tion of the hormones will depend on the objectives of the work, e.g. the use of 2, 4-D should be avoided due to its tendency to suppress morphogenesis (12). If, on the contrary, the aim is to induce cell multiplication without organogenesis, 2,4-D can be included in the medium; thus, 2,4-D in conjunction with 2 iP (a cytokinin) and high sucrose has been used to form callus from cassava stem explants (15); we found that this hormonal combination also promotes callus formation from shoot tip explants of cassava.

On the other hand, plant regeneration from cassava apical meristems was achieved (7) using BAP as a source of cytokinins, NAA as an auxin and GA. IAA is a good source of auxin, but due to its instability and degradation by tissue enzymes (12) it has not widespread use. With certain cassava varieties, 2iP does better than BAP, as a cytokinin, in the differentiation of plants from apical meristems.

We will see later that the proper hormonal balance, the level of sucrose, the surgical manipulations of the cultures and the temperature of incubation all influence the developmental pattern (e.g. single shoot or plantlets, rosette shoots, multiple shoots, etc) of cassava apical meristems.
Hence, these factors can be manipulated to either induce rapid growth and multiplication or slow down the growth rate of the cultures.

The pH of the medium is generally adjusted at 5.3.-5,5 for liquid media and at 5.7 - 5.8 for agar media.

Cassava apical meristems seem to thrive better on Difco purified agar than in Difco Noble agar; the later being a "washed" agar. Agar concentrations from 0.4 - 1.0% have been used. In general, the growth of the meristem increases as agar

concentration decreases. The concentration of agar probably affects the rate of diffusion of large molecules of the culture medium toward or away from the meristem explant, being higher at the lower agar concentrations (2). The concentration of agar may also affect the dilution of inhibitory exudates from the explants. We have noticed that both, through successive (i.e. weekly) transfers of the explants to fresh media and by using large test tubes (i.e. a larger volume of medium) the rate of growht of the meristems is almost doubled.

II. PREPARATION OF STOCK CULTURE MEDIA

a. Preparation of basal medium:

1. The basal medium of Murashige-Skoog (14), slightly modified, is suitable for the culture of cassava meristems.

2. Table 2 presents the constituents of the basal medium grouped into six stock solutions: mineral constituents (stock 1-5) and vitamins (stock 6).

3. The stock solutions should be prepared first, at higher concentrations than the final concentration of the nutrient basal medium:

a. To prepare each stock, dissolve one by one all the ingredients presented in Table 2 in the volumes of water shown at the bottom of Table 2. Use double glass distilled water. Various substances can be combined to minimize the number of stocks; instable substances should be prepared as single stock solutions;chemicals that could precipitate others are not mixed.

b. Stock 5 (the source of iron) is prepared as follows :

Dissolve first the $FeSO_4.7H_2O$ in 50 ml water; then separately dissolve the Na_2EDTA in 50 ml water, heating up slightly; mix both solutions; stir; let cool and make up volume to 200 ml. with water, maintain this solution in the dark.

c. Heat up slightly, in a water bath, any ingredient difficult to dissolve such as some of the vitamins.

d. Stock solutions 2 and 6 should be kept frozen; all others can be kept refrigerated. Prepare stock 6 for use within 1-2 months; all others can be maintained for up to 4-6 months.

4. Prior to the preparation of the basal nutrient medium, allow all stocks to attain room temperature.

5. Table 2 shows the volumes of every stock to prepare one liter of basal medium.

To prepare the basal medium:

a. To prepare a given volume of basal medium, take the required amounts of each stock and pour in a smaller volume of water.

b. Add the required weight of sucrose, dissolve well.

c. Add also the vitamins.

d. Complete to volume with water.

6. The Murashige-Skoog basal medium (MS) satisfies well the mineral salt requirements of cassava meristem cultures. Therefore, for our routine work we found more

TABLE 2. BASAL MEDIUM* FOR MERISTEM CULTURE

Stock No.	Constituents	Composition of Stock ** Solutions(mg)	Final Concentration in Medium mg/l	mM	µM	Volume (ml) of stocks for one Liter Nutrient Solution
1	NH_4NO_3	82,500	1650	20.6		20
	KNO_3	95,000	1900	18.8		
	$MgSO_4 \cdot 7H_2O$	18,500	370	1.5		
	KH_2PO_4	8,500	170	1.25		
2	H_3BO_3	620	6.2		100	1.0
	$MnSO_4 \cdot H_2O$	2,176	22.3		100	
	$ZnSO_4 \cdot 7H_2O$	860	8.6		30	
	$Na_2MoO_4 \cdot 2H_2O$	25	0.25		1.0	
	$CuSO_4 \cdot 5H_2O$	2.5	0.025		0.1	
	$CoCl_2 \cdot 6H_2O$	2.5	0.025		0.1	
3	KI	75	0.83		5.0	1.0
4	$CaCl_2 \cdot 2H_2O$	15,000	440	3.0		2.9
5	Na_2EDTA	1,492	37.3		100	5.0
	$FeSO_4 \cdot 7H_2O$	1,114	27.8		100	
6	Nicotinic Acid	100	0.5			1.0
	Thiamine · HCl	1,000	10.0			
	Pyridoxine · HCl	100	0.5			
	Myo-inositol	10,000	100			
	Glycine	400	2.0			

* Murashige and Skoog medium (1962) modified.

** Dissolve all constituents of Stock 1 in 1,000 ml water; Stock 2 in 100 ml; Stock 3 in 100 ml; Stock 4 in 100 ml; Stock 5 in 200 ml and Stock 6 in 100 ml.

practical to use the pre-made MS mineral salt medium manufactured by "Gibco", in powder form.

Each bag contains 4.3 g of powder to prepare one liter of basal medium; the bags can be kept for about two years under refrigeration and dessicated.

To prepare the basal medium, using the pre-made MS medium:

a. Let the bags to attain room temperature.

b. Weigh the required amount of powder to prepare a given volume of medium.

c. Disolve the powder slowly, in a volume of water smaller than the total volume of basal medium.

d. Add the required volume of the vitamin stock No. 6.

e. Weigh and add the sucrose to the solution, dissolve well.

f. Make up to total volume with water.

The basal medium is now ready for the addition of the hormonal supplements.

b. Preparation of Hormones:

Stock solutions of hormones should be prepared in small volumes for use within 1-2 months only. The stock should be kept refrigerated and should be allowed to reach room temperature before use.

1. BAP (10 ppm) = 6-benzyl-amino purine = 6-benzyl adenine:

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responses of the meristem culture; e.g. regeneration of single shoot and roots, enhancement of axillary shoots, induction of adventitious buds, rooting of shoots, fast or slow growth of the shoots, promotion or inhibition of callus, etc.

The early findings (16) about the morphogenetic stimulus of cytokinin-auxin ratios have greatly influenced the approaches for devising culture media; however, various systems have shown deviations from the general rule.

The responses of cassava meristem cultures to the various hormones, and to other factors of the culture medium, can be summarized as follows:

The cytokinins influence the differentiation of shoots from meristem cultures, low levels (0.01 - 0.02 mg/1) of BAP or 2iP promote the initiation and growth of shoots; five-ten times higher levels, though promoting the initiation, tend to inhibit the growth of both shoots and leaf primordia; at even much higher concentrations (0.2 - 0.5 mg/1) a small "rosette" shoot with several nodes and often with a callus at the base of the shoot, is formed; the amount of callusing depends on the cassava variety. Often, we have observed the initiation of two-four shoots on the upper surface of a callusing meristem explant due to high BAP concentrations. Conversely, rooting is inhibited at the higher cytokinin levels.

The addition of an auxin (e.g. NAA), to a cytokinin-containing medium, further enhances the callusing effect with or without (depending on the variety) rooting. Auxin per se is not essential for the differentiation of shoots in cassava meristem cultures; however, at very low levels (0.02 - 0.05 mg/1) synergistatically acts with high cytokinins in the initiation, but not the elongation, of shoots.

When combined with a cytokinin, gibberallic acid promotes the initiation of single shoots from small apical meristems. In some cassava varieties, GA seems to induce root initiation as well. Higher concentrations of GA, in combination with cytokinins, tend to produce tissue necrosis; BAP being stronger than 2iP.

The sucrose content of the medium also seems to play a role in the organogenesis of cassava meristem cultures. Thus, at low BAP, stem length increases 2-3 fold when the sucrose concentration is increased from 1 to 2% but very little when sucrose goes from 3 to 5%; at higher BAP levels, the sucrose-promoted stem elongation is almost non-negligible at low sucrose, and retarded or inhibited at 4-5% sucrose. In regard to rooting, at low cytokinin levels rooting gradually increases with the addition of one, two and four percent sucrose; however, when the cytokinin level of the medium is high, the addition of sucrose can not overcome the root-inhibiting effect of the cytokinin, even though callusing is promoted. To a degree, sucrose seems to replace the exogenous addition of auxin to the medium; in fact, rooting of "node-bud cuttings", dissected from meristem-derived plantlets, is greatly promoted by sucrose (up to 4%) without the formation of a basal callus; on the other hand, auxin promotes both rooting and basal callusing. In cultures maintained from protacted periods of time, high sucrose levels promote the growth of axillary buds even though the leaves senesce and drop. Too high concentrations of sucrose become detrimental due to osmotic stress and phenolic oxidation in the root system.

The physical conditions of culture interact with the chemical composition of the medium. Exposure of the cultures to low temperature (20°C) counteracts the inhibitory effect of cytokinins on rooting, though shoot growth is retarded.

High sucrose on the one hand, and low temperature on the other, produce very similar stress effects on cassava meristem cultures: promotion of rooting, inhibition of shoot growth and increase in leaf drop, anthocianin development in stems and leaves of some varieties. In a range of varieties, both high BAP combined with low sucrose and low BAP combined with high sucrose induce short, thick, shoots, deep-green leaves and growth of axillary buds.

Rotation of cultures in liquid medium enhances the rate of shoot proliferation. Fig. 3 depicts some of the responses of meristem cultures of cassava to various hormonal combinations and concentrations.

1. Media for Virus Eradication:

Two media have been devised to grow small (0.4 - 0.5 mm) apical meristems of a wide range of cassava varieties. Both media contain the MS mineral salt basal medium, the vitamins, (see Table 2) and 2% sucrose; one medium is supplemented with 0.02 mg/l BAP and 0.05 mg/l GA and the other, with 0.02 mg/l 2iP and 0.05 mg/l GA. If larger explants, i.e. shoot tips are to be cultured, increase the GA to 0.1 mg/l and add 0.05 mg/l NAA. Agar concentration: 0.4-0.6%.

2. Media for rooting:

Terminal and axillary bud cuttings, obtained from meristem-derived shoots, can be planted and roots induced in a medium with half strenght MS salts, vitamins, and 3 -4% sucrose. Agar concentration: 0.8%. Those cuttings comprise the bud plus 1-1.5 cm shoot segment with 1-3 expanding leaves; for smaller (0.5 cm), and not fully differentiated cuttings, the medium is supplemented with 0.01 mg/l BAP.

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3. Media for Propagation:

   a. Media to initiate the cultures: Shoot tips, comprising 3-4 leaf primordia can be induced to form "rosette-type" shoot cultures or "multiple-bud" cultures on MS salts supplemented with the vitamins, 2% sucrose and 0.5-1.0 mg/l BAP; the addition of 0.01-0.02 mg/l NAA promotes the growth of various buds with a basal callus. Agar concentration: 0.6%.

   b. Media to enhance shoot proliferation: The axillary buds formed on rosette plantlet cultures and the buds of multiple cultures can be promoted to grow in rotary shake liquid or stationary agar media: MS salts supplemented with vitamins, 2% sucrose, 0.02-0.05 mg/l BAP and 0.02 mg/l NAA; for solid media, use 0.8% agar.

   c. Media to recover plantlets: Terminal and axillary buds, cut from proliferating shoots, can be planted on the rooting media as at 2 above.

4. Media for the International Exchange of Cultures:

   a. Media to ship single shoot plantlets: Single plantlets can be recovered through the rooting of terminal and axillary bud cuttings from multiple shoot cultures.Use the rooting medium (as at 2 above) with 1% agar.

   b. Media to ship multiple shoot cultures: Proliferating multiple shoot cultures produced as at 3 above, but with 1% agar, can be used for the shipment of larger quantities of materials.

   c. Media to ship shoot-tip cultures: Freshly established shoot-tip cultures can be shipped in media devised to

induce either single (as at 1 above) or multiple (as at 3 above) shoot cultures.

d. Media to handle cultures at the receiving end: Imported cultures are induced to give rise shoots for the excision of terminal and axillary buds; these in turn are rooted as at 2 above.

5. Media for the Conservation of Germplasm:

a. Media to store terminal and axillary bud cultures:Terminal and axillary bud cutting can be stored in the MS salts, without vitamins, supplemented either with 0.02 mg/l BAP and 4% sucrose or with 0.05 mg/l BAP and 1% sucrose. Agar: 0.8%.

b. Media to store multiple- shoot cultures: Upon transfer from the induction media, rosette and multiple-bud cultures can be maintained in the MS salt medium, without vitamins, supplemented either with 0.05 mg/l BAP and 4% sucrose or with 0.1 mg/l BAP with 1% sucrose.Agar:0.8%.

c. Media to recover cultures from storage: Terminal and axillary buds can be cut from stored cultures and planted in the rooting medium (as at 2 above). Small buds could be induced to form rosette or multiple bud cultures.

SECTION D

VIRUS ERADICATION

Viruses, in contrast to insects, mites, nematodes, fungi and bacteria cannot be controlled by the application of chemicals to diseased plants. Viruses strictly depend on living cell's metabolism for their own multiplication. Virus inhibitors tested so far have resulted toxic to the host cells; the virus concentration levels up after the chemical treatment ends (17).

The killing of vectors (insects, nematodes, mites) would alleviate the dissemination of certain viruses, though the dispersal of mechanically-transmitted viruses cannot be controlled by pesticides.

Most viruses are not transmitted through the seed, hence seedlings from infected plants will normally be healthy. However, seedlings or seeds are unsuitable for the maintenance of vegetatively propagated crops such as cassava. Thus, in clonal crops, viruses are transmitted from one generation to the next, with the result that the materials may become completely infected in time; probably all clonal crops are infected with one or more viruses (17) being the latent ones more difficult to detect due to the lack of symptoms.

In a diseased field, luckly a few plants may be found without symptoms. These should be tested for virus freedom.

Routine testing with serology, indicator plants or electron microscopy will confirm the freedom of viruses, especially of the latent ones.

In vitro tissue culture methods have been used to produce virus-free* clones from materials completely infected with one or more viruses (18, 19). The tissue culture methods most commonly used are: meristem and shoot-tip culture, shoot tip grafting and cell and callus cultures to a lesser extent.

The application of the tissue culture-mediated control of viral disease should be integrated with sufficient knowledge about the viral pathogen especially its mode of transmission and host range. The overall virus eradication scheme may then comprise the following steps: a) identification of the causal agent; b) application of the tissue culture technique; c) testing of resultant plants for freedom of the pathogen; d) propagation of disease-free materials under conditions to avoid re-infection.

I. VIRUS ERADICATION AND MERISTEM CULTURE

Since the demonstration of the uneven distribution of viruses in infected plants, and that virus concentration progressively decreases toward the shoot apex (17), a great deal of work was carried out to eliminate systemic viral infections from several economically important crop plants (18, 20). This work has permitted to build up stocks for the production of clean planting materials.

Without detraction of the actual practical usefulness of meristem culture for the elimination of viruses, the basic reasons why this happens are still not completely clarified.

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* The term "virus-free" can be used as long as proper specification is made on the virus in question and on the testing procedures utilized to certify the sanitary status of the plants.

Various hypothesis have been advanced to explain the eradication of viruses by means of meristem culture. Due to the lack of differentiated vascular tissue in the proximity of the apical meristem, virus movement in this region must be slow. This morphological characteristic accompanied by its very active cell multiplication may account for the low concentration or absence of virus in the apical meristem. The latter explanation is supported by the findings (21) that nucleoprotein synthesis required for cell division was more competitive with the synthesis of tobacco mosaic virus in actively dividing tissue cultures than in slow ones.

Some studies (22, 23, 23a) have demonstrated the occurrence of viral particles in the cells of the apical meristem of several species, however, virus-free plants were recovered after culturing the infected meristems. These evidences tend to imply that unknown factors, which probably operate during the in vitro culture stage, as responsible for the loss of viruses in the regenerated plants. A transcient disruption of the apical meristem's normal organization which leads to the inhibition of virus multiplication, due to the unavailability of key enzymes, was proposed to explain the effect of the in vitro culturing stage (25); the disorganization effect may be backed up by the finding (25a) that virus concentration was much lower in disorganized, friable tobacco callus than in compact, organized callus. This was found to be correlated with slower translocation of viruses in the soft callus. Concluding, the culture of tissues in vitro probably affects the normal relationship between virus replication and plant cell metabolism (24). Therefore, any external factor which may affect the plant metabolism can be expected to affect the multiplication of virus within the host.

II. THERMOTHERAPY

Since virus replication seems to be closely related to host cell metabolism, any stress such as low or high temperature affects the replication and movement of virus within the host. Successful thermotherapy depends on the temperature being low or high enough to inhibit virus multiplication, but not too low or too high to prevent host tissue growth. Fig. 4 (25b) shows the general temperature relationship between host and pathogen. The optimal temperature, as well as the duration of the treatment, is related to the particular virus-host association; it is believed in general that isometric and thread-like viruses are more sensible to thermotherapy than rod-shaped viruses (17). Other factors, such as size and water content of the tissue, are also important; eg. viruses from small and well dehydrated tissues and organs such as seeds can be eliminated by thermotherapy (26, 27).

Low temperature, applied to plants prior to meristem culture was more effective than high temperature to eradicate the potato spindle tuber disease (27a), caused by a seed-transmitted viroid.

Although the mechanism of thermotherapy is still unknown, the technique has been used to "cure" many plant crops from viral diseases (20). It has been shown however, that most of these viruses were not eradicated*, but merely inactivated,*

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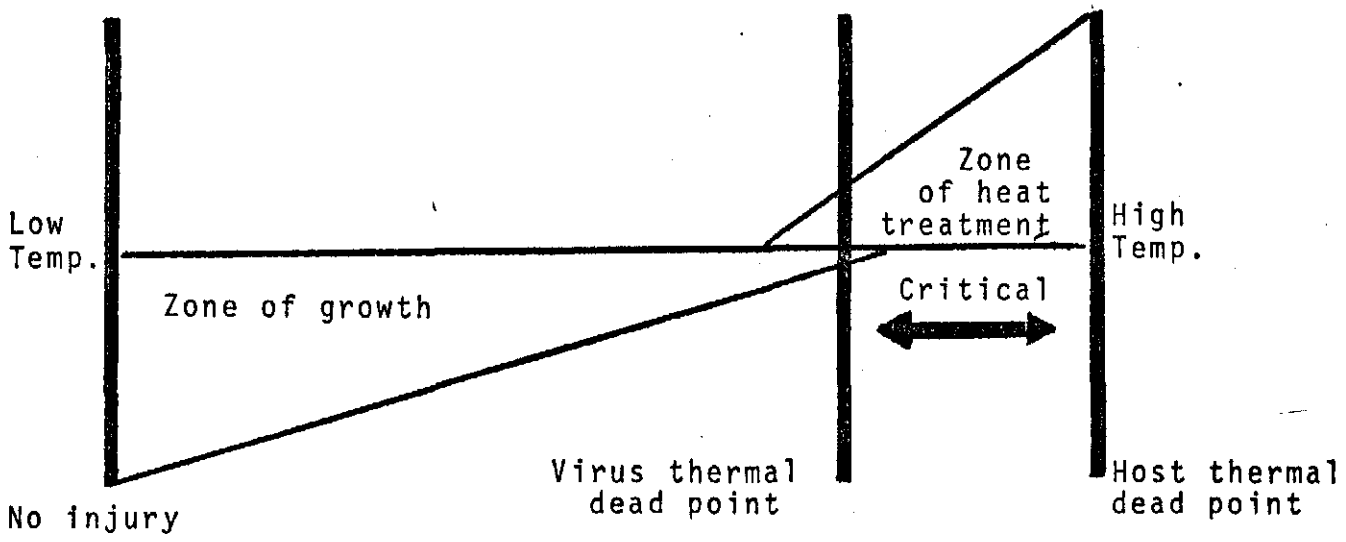
* Following previous work (26), the term eradication (equal to elimination) is referred herein to the complete loss of virus from the host tissue and the term inactivation describes a temporary lack of virus detection by infectivity tests and symptomatology.

FIG. 4 THERMOTHERAPY IN RELATION TO HOST AND VIRUS



(After Baker, 1962) (25b)

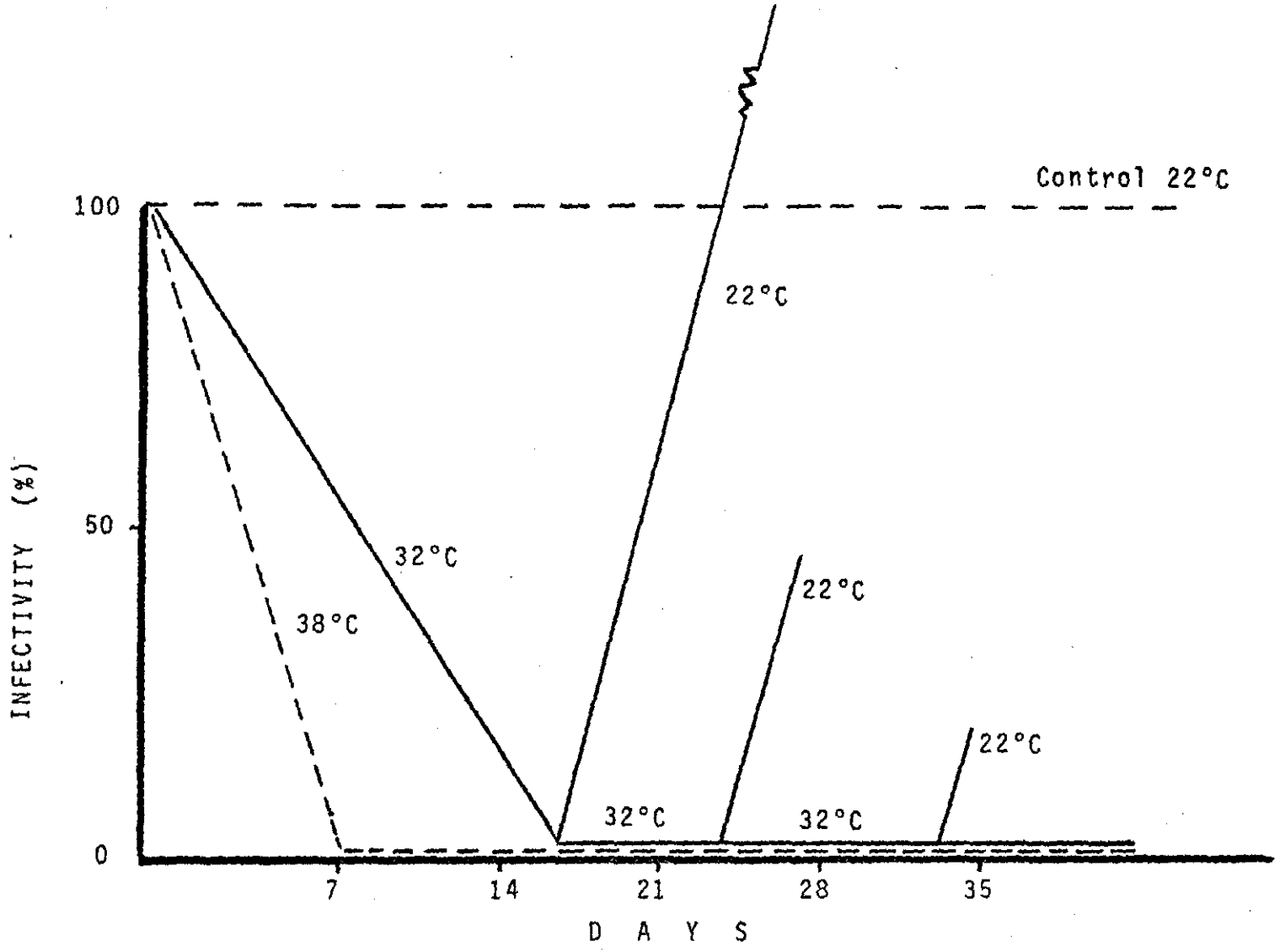
whereby the plants showed a temporary increase in vigor and yield, because later the disease re-appeared (40). For example, newly formed leaves, on infected cassava cuttings, under high temperature (35°C) lack mosaic symptoms; however, upon transfer to a lower temperature (20°C) the mosaic symptoms reappear. Often the re-appearance of symptoms, after short exposures to 32-35°C, is even more pronounced than in leaves continuously maintained at the low temperature.

III. THERMOTHERAPY AND TISSUE CULTURE

Tissue culture methods can be used to study the effects of temperature on virus-host relationships (21). The advantage of tissue culture resides in the experimental material being confined to quite uniform tissues and even to single protoplasts (27b) in which viruses can be replicated almost synchronously (28).

Fig. 5 (29) shows that both the degree and the duration of thermotherapy are important in the inactivation of viruses in tissue cultures. As described above, short periods of mild thermotherapy (32°C) enhance virus infectivity when the cultures are moved to a lower temperature (22°C); only very high (40°C) temperature and much longer treatments can almost completely reduce infectivity; however, this sort of treatment results detrimental to tissue survival. Similarly, virus infected cultures of tobacco and soybean following transfer from short low temperature (10°C) exposures to higher (24°C) temperature showed an increase in enfectivity at even higher levels than in cultures continuously maintained at 24°C (28). Thermotherapy seems to affect the synthesis of viral RNA (30). Successful temperature treatment depends on the ability of host tissues to stand protracted periods of high or low enough temperatures to destroy virus RNA synthesis without impairing host tissue growth.

FIG. 5 HIGH TEMPERATURE INACTIVATION OF VIRUSES



(After Walkey, 1976) (29)

Both cold (27a, 31) and heat (20, 31a) treatments have been widely used to enhance the rate of virus eradication by means of meristem culture. Table 3 shows that the elimination of a wide range of viruses and viral combinations is possible through the appropriate use of heat treatment and meristem culture.

In practice, heat treatment of growing shoots, prior or simultaneous to meristem culture, increases the size of the viruless region of the shoot apex thus, allowing the excision of larger virus-free tips.

IV. CHEMOTHERAPY AND TISSUE CULTURE

The inhibition of virus multiplication by chemotherapy has had limited success. Certain compounds can act as protectants against the infection of viruses (32) and others, such as purine and pyrimidine base analogs, may actually interfer with viral RNA synthesis (17); however, these substances are also toxic to the host cells (21). Ribavirin, a compound with broad anti-viral activity against both DNA and RNA containing viruses (33) was used, in conjunction with tissue culture, to eradicate potato virus X (34); only shoot forming cultures became free of the virus.

V. OTHER TISSUE CULTURE SYSTEMS

Leaves infected with mosaic, often present small patches of green mesophyll tissue. These "dark-green islands" can be isolated and cultured *in vitro* to give rise to plants; high rates of virus-free plants have been produced by this method in tobacco (35). For reasons still unknown, some of the cells of a dark-green island are resistant to viral invasion. Protoplast culture in conjuction with chemotherapy has also been used to

TABLE 3. Production of disease-free Solanum species infected with single viruses and with double or triple virus combinations by means of thermotherapy and meristem culture.*

Species \ Viruses	No. of Varieties cleaned-up and Eradication Rates(%)**						
	PVX	PVS	PVX+ PVS	PVX+ PVY	PVX+ APLV	PVX+ PVS+ PVY	Total
1. adg.	6(50)	2(60)	1(50)	3(90)	1(100)	1(33)	14(64)
2. tbr.	11(66)	2(62)	5(64)	5(55)	-	-	23(62)
3. phu.	1(90)	-	-	-	-	-	1(90)
4. juz.	2(100)	-	-	-	-	-	2(100)
5. tbr. x adg.	3(61)	-	2(90)	1(35)	-	-	6(62)
6. tbr x phu.	2(90)	-	2(95)	-	-	-	4(92)
7. tbr. x neotbr.	-	1(60)	-	-	-	-	1(60)
TOTAL	25(76)	5(50)	10(75)	9(60)	1(100)	1(33)	

** Percent eradication appears in parenthesis to right of the No. of varieties.

* (W.M. Roca, unpublished)

eradicate certain viruses (34).

Successful elimination of various citrus viruses has been achieved by grafting *in vitro* shoot tips of infected plants onto disease-free rootstock seedlings; in this procedure, the pathogen-free plants showed no reversion to the juvenile phase (35a) as occurs with the plants grown from nucellar tissue.

VI. VIRUS ERADICATION IN CASSAVA

In spite of the amount of work carried out on cassava mosaic disease (CMD or African Mosaic) (36, 37) the exact nature of the causal agent still remains unknown (38). However, previous work has demonstrated that healthy (i.e. symptom free) plants can be produced by meristem culture in cassava (27). Heat treatment, prior to meristem culture, permitted to double the size of the meristem explant (from 0.4 to 0.8 mm) without loss in the efficiency of disease elimination.

Other viral diseases reported in cassava are: the common mosaic (CCM), brown streak (CBSV), vein mosaic and cassava latent virus (CLV) (38a).

The mosaic symptoms of a cassava variety, probably infected with common mosaic, disappeared after a three-week heat treatment (35°C). We have produced healthy clones of this variety through meristem culture of heat treated plants.

Another viral-like disease in cassava, characterized by the occurrence of lignified cracks along the roots, and often associated with significant reduction in tuber yield (39), has been named "frog-skin" disease. Our research with the "frog-skin" disease indicates that high temperature (35 - 40°C) treatment of infected stem cuttings, prior to the culture of small apical meristem explants, resulted in eradication rates of around 90%.

Interestingly, similar rates of eradication have been obtained by successive sub-cultures of the meristem. Work is underway to use heat treatment directly applied to meristems during culture.

VII. MERISTEM CULTURE TECHNIQUE FOR THE ELIMINATION OF VIRAL DISEASES IN CASSAVA

The main steps in the methodology for virus elimination from cassava, is shown in Fig. 6.

a. Thermotherapy:

1. To prepare the plant materials for thermotherapy follow the procedures described in Section A, I:1-6.

2. Conditions of thermotherapy:

   Temperature: 35°C day and night until the sprouts are about 2 cm high (10-15 days). Then increase the day temperature gradually (1°C a day) up to 40°C.
   Photoperiod: 16 hour day and 8 hour night.
   Illumination: 1,000 - 2,000 lux
   Humidity: Water the pots as needed to maintain high humidity.

b. Preparation of Culture Media:

To prepare the basal medium (MS), see Section C, IIa:1-6 or 6a-6f.

Formulation of media:

1) MS (2% sucrose) + 0.02 mg/l BAP + 0.05 mg/l GA
2) MS (2% sucrose) + 0.02 mg/l 2iP + 0.05 mg/l GA

Preparation (500 ml of each medium):

1) Pour about 400 ml of MS basal in each of two beakers. Label 1 and 2.

2) Dissolve 10 g sucrose in each beaker.

3) To calculate the volume of the hormonal supplements, use the formula: $C_1V_1 = C_2V_2$, where :

   C_1 = concentration of hormone stock = 10 mg/l;

   V_1 = ml of each hormone stock needed to make up the final concentrations, as shown in the formula;

   C_2 = final concentration in each mediun = 0.02 mg/l BAP or 2iP and 0.05 mg/l GA;

   V_2 = final volume of each medium = 500 ml.

   Thus, $x = \frac{0.02 \times 500}{10} = 1.0$ ml BAP or 2iP

   $x = \frac{0.05 \times 500}{10} = 2.5$ ml GA

4) Add to medium 1: 1.0 ml BAP and 2.5 ml GA and to medium 2: 1.0 ml 2iP and 2.5 ml GA.

5) Complete to 500 ml volume with glass distilled water.

6) Adjust pH: 5.7 - 5.8.

7) Add 0.6% agar = 3.o g agar/medium: heat up the media in a water bath until the agar is completely dissolved.

8) Distribute each medium in small test tubes (100 x 12.5 mm), let the tubes cool slightly and cap.

9) Autoclave the media at 15 lb./in^2 (121°C) for 10 minutes. Allow slow de-compression.

10) Let the media cool at room temperature and then store at 6 - 10°C.

11) To prepare the sterile tissue and to isolate the apical meristems follow the procedures described in Section A, II:1-3 and IIIc:1-11.

12) Incubation of cultures: See section A, IV:1-3.

13) For the hardening of cultures, potting and field transplanting, see Section A: V, VI, and VII respectively.

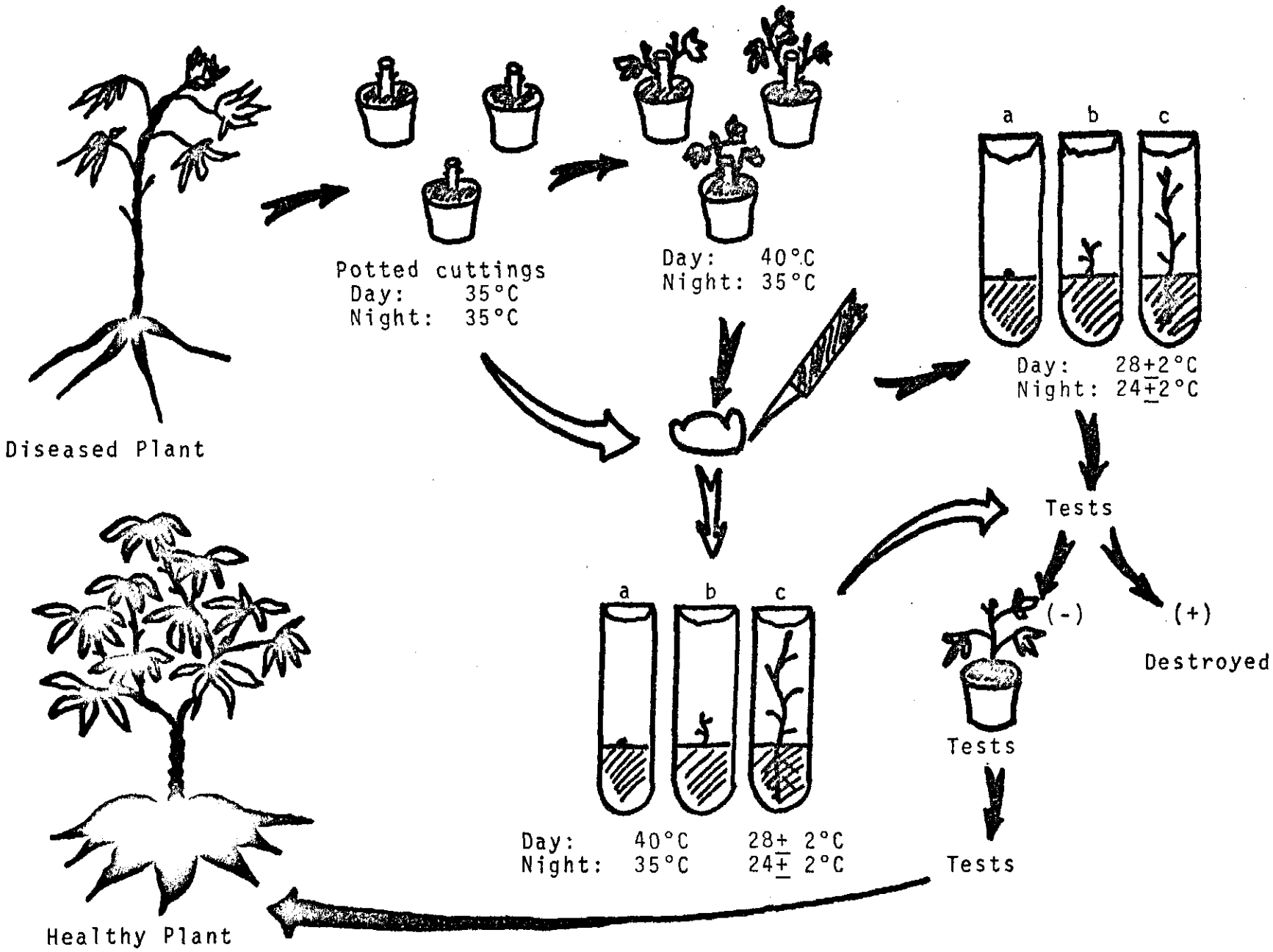


FIG. 6 Main steps in the technique for disease eradication in cassava by thermotherapy and meristem culture.

SECTION E

PROPAGATION BY MEANS OF MERISTEM CULTURE

In the last decade, tissue culture methods have become increasingly important as a profitable alternative in the propagation of various plant species (8). Due to the initial sucess with ornamentals (41), the meristem and shoot-tip culture techniques have been incorporated into the routine activities of several commercial nurseries.

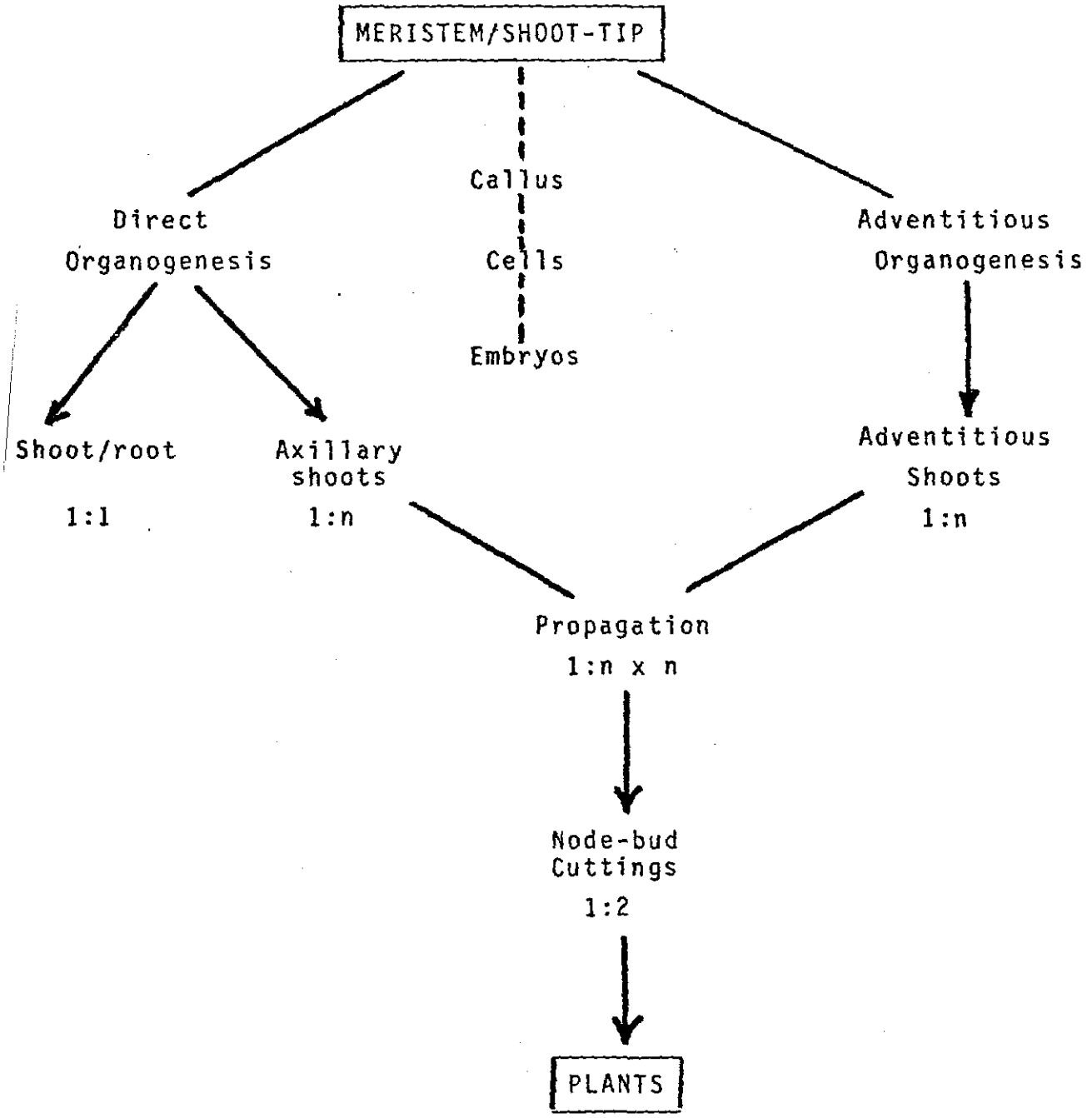
Along with the elimination of viruses (see Section D) meristem culture, when properly used, provides a means to rapidly multiply valuable specimens and to propagate difficult-to propagate species in a continuous fashion. In contrast to other tissue culture systems, meristem propagation or "mericloning" maintains high stability in the genotypic features of the plant material (42); this is a highly desirable attribute for vegetatively propagated crop plants; along with this, meristem and shoot-tip cultures provide means to maintain the cultures with a minimum loss of organogenic potential (i.e.high totipotency).

I. SYSTEMS OF MERISTEM CULTURE PROPAGATION

Fig. 7 shows some of the possible routes of plant asexual propagation that could be achieved through modulation of meristems or shoot-tips explants during culture in vitro.

The simplest route involves the growth of a rooted plantlet from an isolated meristem explant (direct organogenesis). Due to its 1 : 1 propagation rate, this system can be used for the recovery of virus-free stocks or to propagate difficult species. Slight increases in propagation rates (1:2-4)can be

FIG. 7 PROPAGATION OF PLANTS BY MEANS OF MERISTEM CULTURE: POSSIBLE SYSTEMS.



achieved by the culture of stem segments, which contain one node-bud each, cut from the single meristem-derived plantlet (42).

A more widespread propagation system consists in the promotion of growth of many buds from a shoot-tip (8).In this system, the growth of the original shoot-tip explant is kept to a minimum; instead the axillary buds which could have been present in the explant plus those newly formed ones, due to the culture stimulus, grow into multiple shoots. The number of shoots produced per shoot-tip explant and the potential to regenerate more shoots through sub-cultures depend on the culture conditions and the plant species. Large scale propagation has been achieved by this method with some plants (40); commonly a cytokinin triggers the continuous production of axillary shoots.

Other systems, whereby adventitious buds and shoots are induced on an "organogenic callus" formed from a shoot-tip (5,42,45) has yielded high multiplication rates. The original shoot-tip explant remains latent while a green, hard, callus develops around it as a consequence of the culture stimuli; then "meristemoids", which arise in the callus, differentiate into buds and eventually into shoots.

Much higher multiplication rates could be achieved by putting to work together the last two systems. Adventitious shoots, induced on organogenic cultures, could in turn be used to proliferate axillary shoots. Node-bud cuttings could then be cut from these shoots and planted on agar media to recover entire plants.

In order to develop a propagation system, perhaps in a commercial level, every step along the process should be monitored to obtain the highest yields; eg. the source of meristem explants, the conditions of culture, laboratory, greenhouse and

field space, technical staff, equipment, etc.

II. PROPAGATION OF CASSAVA BY MEANS OF MERISTEM CULTURE

Cassava, being a highly heterozygous crop, is preferentially propagated by vegetative means. However, due to both the long growth cycle of the crop and the very few cuttings available per stem, the rates of propagation obtained by conventional techniques are quite low. This is even more critical, considering the future need of large numbers of uniform planting materials for use in energy oriented cassava cropping.

Meristem culture propagation of cassava could be more profitably used within a practical sheme on maintenance and distribution of germplasm in disease-free conditions. Promising breeding selections could be quickly multiplied by shoot-tip culture and stem cuttings (44), thus, materials free of unacceptable diseases could be distributed for the regional trials; likewise, germplasm could be processed before distribution.

Previous work with cassava meristem culture (7) failed to exploit the potential of the technique as a vehicle for rapid propagation. Preliminary results of our research (43) suggests that cassava can be amenable, through simple culture manipulations, to high rates of multiplication. The technique, as it stands now, basically consists in the inhibition of cassava's natural strong apical dominance in order to induce "rosette-type"cultures bearing each 10-15 nodes. Each node bears an axillary meristem which has the potential of reproducing the shoot. Upon transfer of the rosette cultures to media devised to promote the growth of such buds, 6-10 shoots can grow per rosette culture. Thus, "multiple shoot" cultures

can be maintained through 4-5 successive subcultures. Entire plants can then be recovered by the culture of node-bud cuttings obtained from the multiple shoot cultures. Fig. 8 depicts the main events of the multiple shoot culture system in cassava. Further research along these lines should provide much higher multiplication rates by means of a proper combination of adventitious and axillary shoot production.

III. PROPAGATION TECHNIQUE IN CASSAVA

a. Induction of Rosette Cultures:

1. Prepare the plant materials as at Section A, I:1-6.

2. To prepare the basal medium (MS) see Section C, IIA: 1-6 or a - f.

3. Formulation of media for culture initiation:

   1) MS (2% sucrose) + 0.5 mg/1 BAP + 0.01 mg/1 NAA.

   2) MS (2% sucrose) + 1.0 mg/1 BAP.
      Agar:0.6%.

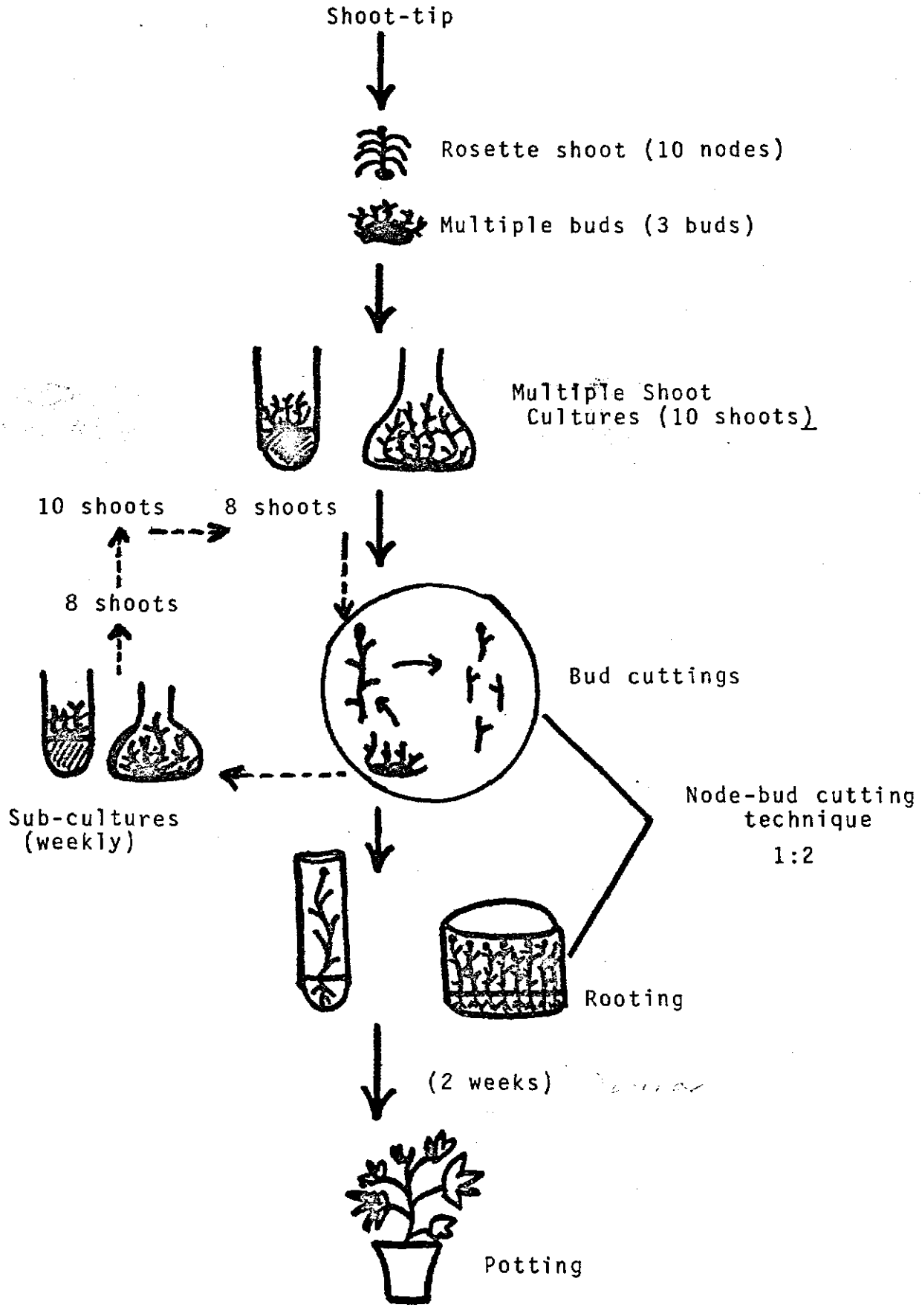
4. For the preparation of these media, follow similar steps as described in Section D, VII b: 4-13.

5. Sample the sprouts, desinfest, and excise the shoot tip explants as described in Section A, II: 1-2 and IIIc: 1-11.

6. Incubate the cultures under the conditions described in Section A, IV: 1-3.

7. After 3-4 weeks of culture, a short rosette-type,

FIG. 8 MERISTEM PROPAGATION OF CASSAVA THROUGH THE MULTIPLE SHOOT CULTURE AND NODE-BUD CUTTING TECHNIQUES.



shoot developes in most varieties; some others may form a small callus with various bud initials on its upper surface.

b. Proliferation of shoots:

1. Remove all the expanded leaves and the basal callus from the rosette cultures and transfer these to the proliferation media:

   MS (2% sucrose) + 0.02 mg/1 BAP + 0.02 mg/1 NAA. Agar: 0.8%, or
   MS (2% sucrose) + 0.05 mg/1 BAP + 0.02 mg/1 NAA. Liquid, rotary shaker.

   For the preparation of these media, see 4. above.

   If agar media, place horizontally on the agar one rosette culture per tube (150 x 25 mm) containing 10 ml of medium. If liquid media, transfer 3 rosette cultures per 250 ml flask containing 15 ml of medium.

2. Within two weeks, the liquid cultures have formed various shoots per explant; the agar media will normally take one more week.

3. Once the shoots have attained 1.5-2.0 cm length, they can be cut off from the proliferating mass and planted as terminal bud cuttings on the rooting medium.

c. Recovery of Plantlets: The node-bud cutting technique:

1. Plant the terminal (and axillary, if present) bud cuttings (see Fig. 8) on the following medium:
   1/2 strength MS (4% sucrose). Agar: 0.8%

2. Within 7-8 days, root initials have formed at the basal end of each cutting.

3. Treat the cultures through the hardening process (see Section A, V: 1-3).

4. After about two weeks hardening, the plantlets can be moved to the greenhouse for potting (See Section A, VI: 1-8).

5. Following 3-4 weeks, the potted plants are ready for field transplantation (See Section A, VII: 1-9).

Steps 8-13 can be repeated as many times as possible, depending on the organogenic potential of the proliferating cultures, through successive sub-cultures.

SECTION F

INTERNATIONAL EXCHANGE OF GERMPLASM BY MEANS OF MERISTEM CULTURE

The movement of plants or plant parts from one country or region to another plays an important role in the transfer of technology carried out by national and international agricultural organizations. However, this function is very often restricted by regulations which prevent the introduction of new germplasm, especially of vegetatively propagated crops, because of the real risks of disseminating pests and diseases (46,47). Such concern has been thouroughly reviewed in a recent book on plant quarantine in the transfer of genetic resources (48).

Due to recent advances in the technology of tissue culture to free plants from diseases, it is now possible to utilize these methods for the international exchange of clonal materials in disease-free condition or to transfer germplasm from one country where organisms of quarantine significance exist to another where they do not (46, 50, 51). Indeed, tissue culture meets with a basic quarantine principle: the smaller the consignment the lower the risks; another advantage is the freedom from contaminating organisms in tissue cultures since the system has a built-in capability for detecting pests and pathogens.

Cultures initiated from 0.5-0.7 mm meristem tips, in an artificial nutritive medium, should be free of insects, nematodes and most fungi and bacteria (50).

Should these be present in the original explant , they could be detected in culture. In the case of obligate parasites, such as viruses, the cultures for shipment should be initiated from stocks free of viruses (Section D). This procedure should provide adequate safeguards to prevent the dissemination of dangerous pests and diseases across country or region boundaries.

MERISTEM CULTURE TECHNIQUE FOR THE EXCHANGE OF CASSAVA GERMPLASM

The overall transfer process may comprise the following steps:

I. Production and Multiplication of Disease-Free Clones.

II. Preparation of Cultures for Distribution.

III. Evaluation, Packing and Shipment of Cultures.

IV. Handling of Cultures at the Receiving End.

V. Release of Materials.

I. PRODUCTION AND MULTIPLICATION OF DISEASE-FREE CLONES

a. Materials infected with viral diseases should first be cleaned up. Follow the procedures described in Section D, VII. Reliable indexing techniques should always be used to prove the cleanness of the clones.

b. Once the materials have been freed from viruses, rapidly multiply to build up stocks for distribution. For propagation see Section E, III. Attention should be given to maintaining sterility of the cultures throughout the propagation steps.

II. PREPARATION OF CULTURES FOR DISTRIBUTION

The type of culture to be prepared for distribution is greatly related to the recipient institution or country, especially in regard to the facilities for handling the cultures.

Cassava clones could be distributed in one or more of the following culture types:

a. Single rooted plantlets.

b. Shoot-tip cultures

c. Multiple-shoot cultures.

a. Single rooted plantlets:

This is the culture type requiring the simplest handling at the receiving end.

1. Cut terminal or axillary buds with a portion of stem (node-bud cutting) from multiple-shoot cultures produced as in Section E. For the node-bud cutting technique see Section E, c: 1-2.

2. Plant the cuttings on the surface of the following media:

   1/2 strength MS(4% sucrose).

3. Place one cutting per each, 100 x 16 mm, test tube. Use 1% agar and test tubes with a constriction at the lower third in order to prevent the agar getting mixed up with the plantlet during shipment. Two-three cuttings could be planted in larger tubes (100 x 25 mm).

4. Cap the tubes and place them in the incubation room (see Section A, IV).

5. In about one week roots have been initiated.

6. Transfer the cultures to the hardening phase (see Section A, V).

b. Shoot- tip Cultures:

This culture type allows to ship at least 10 shoot tips per test tube.

1. Excise shoot-tips (See Section A, III) and culture in media devised to induce either single (see Section D, b) or multiple-shoot (see Section Ea: 1-6) cultures. Increase agar to 0.8%.

2. Incubate the culture as above for 1-2 weeks. After this period of time, the cultures are ready for shipment.

c. Multiple-shoot Cultures:

This culture type permits the shipment of many buds or shoots growing on proliferating structures. Such cultures require more specialized handling at the receiving end.

1. Excise shoot-tip from disease-free clones and culture in media to induce rosette cultures (see Section Ea:1-7).
2. After 3-4 weeks, transfer the rosette cultures to the proliferation media as at Section Eb: 1-2. Use only agar (0.8%) media.

3. In about 1-2 weeks, the axillary buds of rosettes have grown to a stage suitable for shipment.

To prepare the plant materials as well as for the surface sterilization and aseptic isolation of explants follow the procedures as at Section A : I, II and III, respectively.

The size of the vessels for shipment should, in the initial trials of exchange, be small (eg.100-150 x13-16mm test tubes). After enough confidence in the system has been built up, larger vessels (made of plastic or aluminum)could be used; each of these may hold up to one hundred shoots or plantlets.

III. EVALUATION, PACKING AND SHIPMENT OF CULTURES

a. Evaluation:

1. The evaluation refers to the phytosanitary observations and tests that should be carried out on all cultures prior to packing and shipment. As discussed earlier, any fungal or bacterial contaminants should be readily noticed after 2-3 weeks incubation, since the media is so rich that it should be able to support the grow of such organisms.

2. In the case of doubt or when the presence of a fastidious organism is suspected, special media may be de - vised to support their grow to render it visible.

3. If a viral-type disease is suspected the available virus testing methods should be used.

4. Only the cultures which show no contaminations should be packed for shipment.

b. Packing and Shipment:

1. The use of polystyrene containers can protect the cultures against changes of temperature. In a recent airmail shipment to Brasil we have recorded a minimun of 20°C and a maximum of 43°C, without irreversible damage to the cultures.

2. Abrupt changes in atmospheric pressure in the plane's mail compartment may also be a cause for damage. The use of partially pressurized compartment in the plane is advisable.

3. Agar may become disturbed due to shaking; this can be overcome by using specially made test tubes (as described above) and high (0.8-1.0%) agar media.

4. Darkness will undoubtly affect the cultures. They will turn pale-yellowish and slender due to etiolation.Both, the choice of the most direct airway as well as a prompt clearance from customs will facilitate the recovery of the cultures at the receiving end.

5. The cultures could also be hand-carried. Whenever possible, this system would be the safest.

6. Every shipment should be accompanied by a detailed list of clones, a phytosanitary certificate issued by the competent authorities and a step by step protocol on how to handle the cultures on receipt if necessary.

IV. HANDLING OF CULTURES AT THE RECEIVING END

Undoubtly, the most critical aspect in the international transfer of cultures is the recovery and propagation of plants after their arrival to the recipient country. It was demonstrated (50) that appropriate training of personnel from recipient countries on the techniques for recovery, testing and propagation of materials is a very important aspect of the overall program.

Every culture type, as described above, will require a special handling technique once it has arrived to its destination:

a. Single rooted plantlets

1. After their arrival, expose the cultures to low (1000-2000 lux) diffuse illumination in order to re-green the cultures, for about 5-7 days.

2. If facilities allow, subject the plantlets to a hardening treatment as at Section A, V.

3. After about two weeks, the plantlets are ready for potting (see Section A, VI).

4. Should any culture arrive distrubed, it could be saved by culturing node-bud cuttings from what remains of the shoot (see Section E, III, c).

5. If facilities allow, and if the plantlets have visible axillary buds, 2-5 plantlets could be recovered from each shoot through the node-bud cutting technique (see Section E, III,c).

b. Shoot-tip Cultures:

The handling of shoot-tip cultures, and multiple-shoot cul-

tures (see c. below), require more specialized facilities than the single rooted plantlets:

1. After arrival, allow the cultures to green under diffuse lighting for about one week.

2. Transfer each shoot tip culture to individual tubes containing medium to induce either single (see Section D, b) or multiple (see Section Ea: 1-6)cultures. The latter should always be prefered in order to enhance propagation.

3. After the rosette cultures have formed, transfer to the proliferation media (see Section E, IIIb: 1-3).

4. Once the shoots are 1.5-2.0 cm long, recover plantlets by using the node-bud cutting technique (see Section E, IIIc: 1-2).

5. Harden the plantlets as at Section A, V.

6. Potting as at Section A, VI.

c. Multiple-shoot cultures:

1. Place the cultures under diffuse lighting for about one week.

2. Transfer to higher illumination (3,000-4,000 lux) to enhance the growth of shoots.

3. Recover plantlets by means of the node-bud cutting technique (Section E, IIIc: 1-2).

4. Harden the plantlets as at Section A, V.

5. Potting as at Section A, VI.

The advantages of the multiple-shoot culture system are: many potentially vigorous plants can be shipped per unit vessel; in a 100 x 13 mm test tube a 5-6 shoot culture can be shipped; relatively simple handling at the receiving end; and allow the build up of large numbers of clones from the importation of a few cultures since through successive sub-cultures, shoot-proliferating structures can be maintained.

V. RELEASE OF MATERIALS

Quarantine regulations vary from country to country; from those where vegetative materials are readily accepted to those where their introduction is forbidden.

Because the center of origin of cassava may also be a center of diversity for certain pests and diseases, several countries have erected strict regulations which ban the introduction of cassava vegetative materials. The main cassava pests and diseases have been described (49) in relation to their quarantine significance in various regions of the world.

Tissue culture transfer of cassava should alleviate such restrictions and eventually provide a very safe system for the introduction of valuable germplasm in clonal form. It has been demonstrated (50) that the technique can be applied to that end.

In the case of countries where certain pests and diseases do not exist, the strategy could be to create an indetermediary recipient location where the initial testing and propagation could take place. Tested materials would then be re-distributed to the other countries of the region.

1. The potted plantlets as at IV above, can be ready for random sample testing in order to confirm the freedom from diseases.

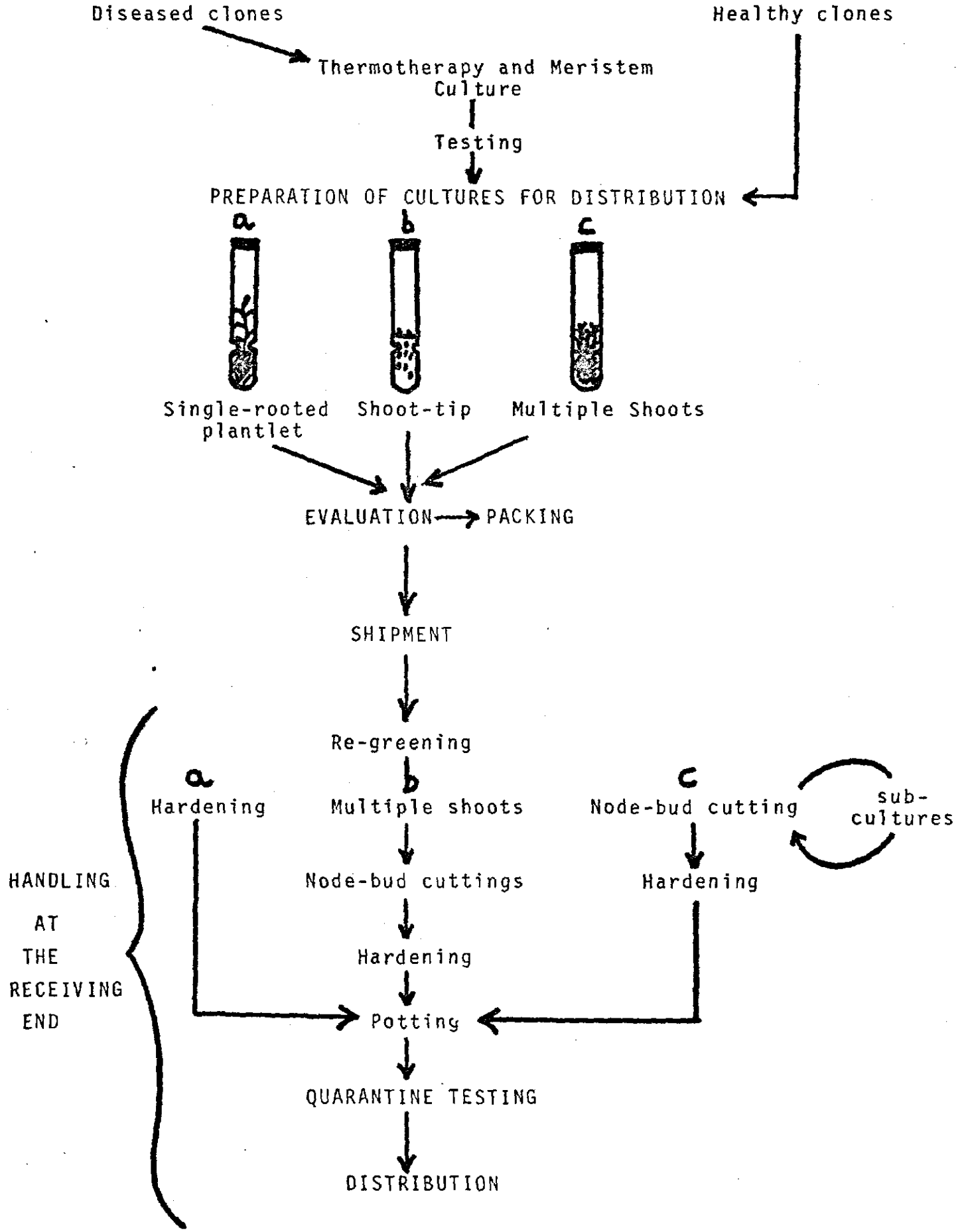
2. Tested clones can be propagated either or both by *in vitro* techniques (see Section E) or by more conventional methods, such as stem cuttings.

3. Vegetative materials can be taken to the field or redistributed to other countries of the region in the form of: a. potted meristem-derived plants; b. rooted stem cuttings; c. tissue cultures.

The system to be used will depend on the requirements of each country's regulations and on the facilities to handle each of the systems.

Fig. 9 outlines the various steps in the use of meristem culture for the international exchange of cassava germplasm.

FIG. 9 INTERNATIONAL TRANSFER OF CASSAVA GERMPLASM IN THE FORM OF MERISTEM CULTURES



SECTION G

CONSERVATION OF GERMPLASM BY MEANS OF TISSUE CULTURE

Because of modern agricultural practices, the genetic variability of crops is gradually diminishing, thereby, valuable genetic resources are being lost. The need for sources of resistance to pests and diseases as well as other agronomic traits justifies the availability and preservation of germplasm collections.

Long-term storage of germplasm is expensive. Work is needed to make storage procedures more efficient and less expensive; eg. the effect of temperature, moisture, atmosphere, etc. on the longevity of seeds warrant study (52); research is needed on cryogenics of clonal tissues as well as pollen, etc.

Germplasm can be stored in various forms: seed, pollen, vegetative organs for short-life perennial crops and field plantations for long-life perennial crops (53).

It is imperative that any storage system should guarantee minimal losses and maximal genetic stability (54). In most plants, seeds can meet these requirements; seeds under ideal conditions can remain viable for up to many decades (55). With a life span shorter than seeds, pollen storage can also be used in certain cases (55).

Vegetatively propagated crops require that high levels of heterozygocity be preserved (which otherwise can be lost trough seeds or pollen), thus, vegetative organs are prefered to maintain valuable gene combinations. However, in most clonal crops, the perennation through vegetative organs requires con-

tinuous field cultivation; this in turn demands intensive labor and is expensive. Furthermore, the risks of losses due to pests and diseases are high.

The potentially high propagation rates that can be achieved with tissue cultures coupled with their freedom from microorganisms and small space requirements make it feasible to utilize these procedures for the conservation of germplasm of vegetatively propagated crops.

The tissue culture storage method should meet certain requirements to be utilized in practice: the system should secure high genotype stability and high propagation rates of the materials so as to quickly retrieve and reproduce true-to-type clones from storage, when needed.

I. THE PROBLEM OF GENOTYPE STABILITY

Specially important in tissue culture storage is the requirement for high stability of the clone's characteristics.

Plant cell and tissue cultures either of somatic (non-meristem) or germinal (pollen) origin seem to display unstability (56) due to either a carry-over effect of the *in vitro* variability or due to changes induced during the *in vitro* process. Both euploidy and aneuploidy have commonly occurred in those cultures (56); in fact cell protoplast and callus cultures have been proposed as effective means to generate variant (either spontaneous or induced) with certain desirable crop attributes (57).

In contrast, meristem and shoot-tip cultures provide effective means to maintain high genotype stability (42). Meristem cultures own their innate stability to the strict control of the sequence: DNA synthesis-mitosis, which does not allow extra du-

plication of DNA (i.e. somatic polyploidy). The continuous cell division in the meristem as a whole also tends to eliminate some of the chromosomal aberrations and other genetic defects (56).

Because of these reasons, plus the elimination of pathogens, meristem cultures should be prefered as a method to store germplasm in clonal form. In addition, meristem culture, in contrast to cell and callus cultures, possess high organ forming capacity during extended periods of culture.

II. TISSUE CULTURE STORAGE METHODS

a. Freeze-preservation

b. Serial transfer of cultures

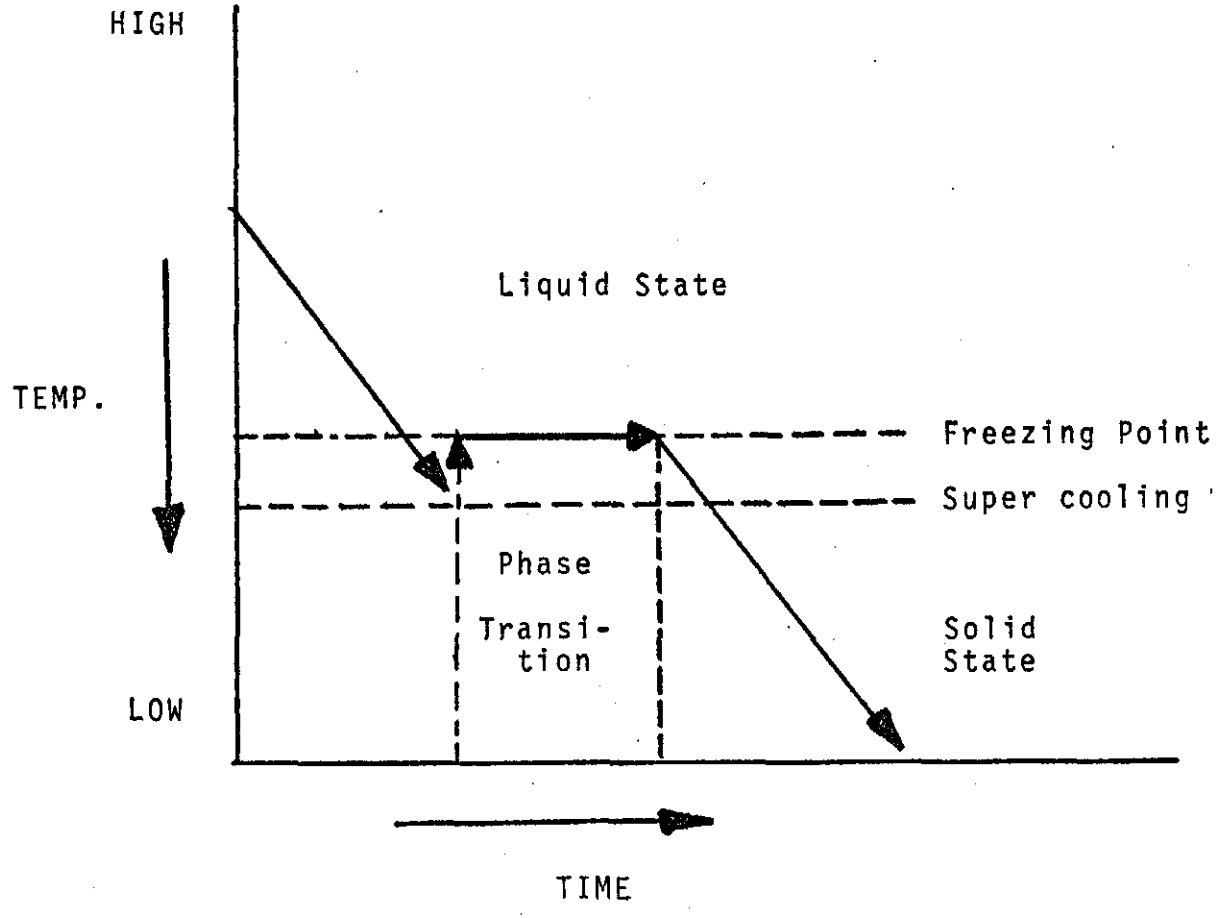
c. Preservation under minimal growth conditions

a. Freeze-preservation:

To a certain degree in most tissue culture storage systems, a danger of genetic impairment of the materials exists over extended periods of storage. Conservation of cultures under sub-zero temperatures may reduce such risks. The ultimate condition, under which normal thermal-driven reactions may be totally arrested, is the storage under liquid nitrogen temperature (-196C); only background radiation, which can occur after hundreds of years, may damage the cells (58).

Fig. 10 shows some of the heat-transfer relationships between biological materials and the cool milieu (59). When a cell is subjected to sub-zero temperatures it initially supercools, regaining thermodinamic equilibrium either by transfering water to the exterior or by intra-

FIG. 10 COOLING DIAGRAM SHOWING THERMAL RELATIONSHIPS BETWEEN BIOLOGICAL MATERIAL AND THE COLD MILIEU.



(After Bajaj, YPS and Reinert, J. 1977)(59)

cellular freezing. Water will move to the exterior, avoiding intracellular freezing, if the permeability of the membrane is high enough or if the cooling rate is slow enough. However, if water leaves the cell too quickly, the increasing concentration of salts inside the cell will become damaging.

Therefore, the factors which may lead to cellular damage during cooling to sub-zero temperatures are: intracellular ice-crystal formation, increase in the concentration of intra or extra cellular solutes and denaturation of proteins as a result of dehydration.

1. Freeze-preservation of cells and tissues:

Successful freeze-preservation of cell suspensions and recovery of plants through embryogenesis was achieved in carrot (60), likewise, though without organogenesis, work was done with cell suspensions of sycamore (61) and flax (62). It was shown here that higher cell survival was correlated with the meristematic activity of the tissue; the larger, more vacuolated cells were mostly damaged. Growth, after retrieval from liquid nitrogen, only occurred in those meristematic areas.

Recently, carrot embryos and even plantlets have been successfully preserved in liquid nitrogen (63); it was found that tissue dehydration before or during freezing increased survival after thawing, conversely rapid thawing was necessary when the tissue was dehydrated to a lesser degree. Embryos gave rise to new growth through secondary embryogenesis while in the plantlets, the root and shoot meristems gave rise to new growth.

Besides the control of the cooling rate, other factors which influence the survival of cultures after freeze-thawing are related to the age, nature and density of the cell cultures (59). Thus, meristematic cells, low free-water content of the cells, high density of cells, single cells rather than callus masses and the degree of cold hardiness, all favor the rate of survival.

There seems to be a critical temperature for the growth of lethal ice crystals within the cells (64). Lethal ice crystal growth could be prevented by rapidly passing the tissue through the critical lethal temperature (around - 70°C). Fig. 11 (64) compares the cooling curve for fast (a) and slow (b) cooling rates. Similarly, fast rewarming of the cells (c) during retrieval from liquid nitrogen prevents the growth of ice crystals due to re-crystalization.

Hence intracellular ice formation per se is not as important to cell survival as the actual size of the crystals; these should remain small enough to prevent irreversible damage.

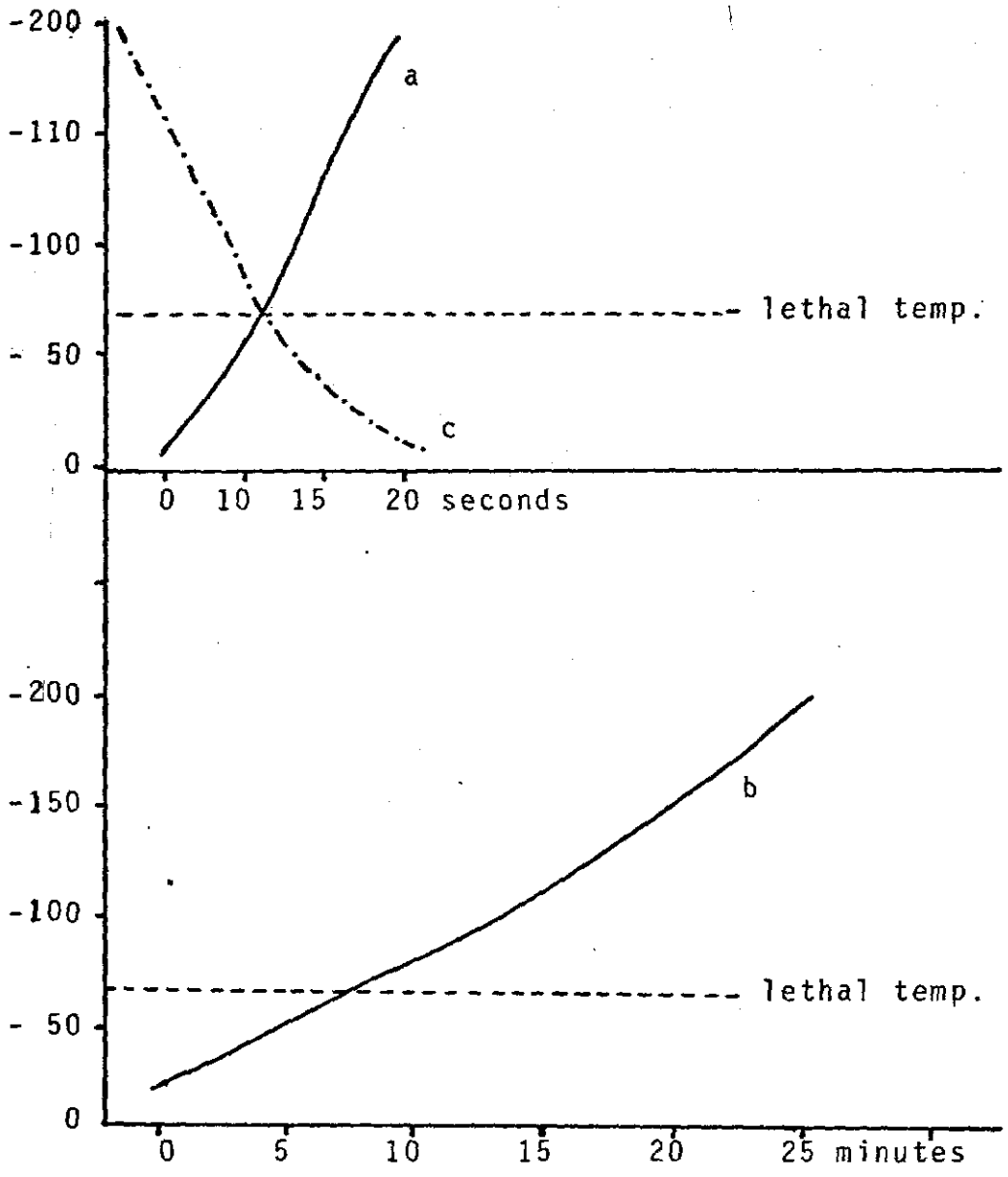
The treatment of tissues, prior or during freezing, with physiological (eg. sucrose) or non-physiological (eg. glycerol, dimethyl sulfoxide) substances probably act by decreasing the growth of ice crystals and thus prevent cell damage. Fig. 12 (60) shows the effects of various cryoprotectants on the percent survival of carrot cells after storage in liquid nitrogen.

2. Freeze-preservation of meristems:

Even if cells survive and divide after retrieval

LIQUID NITROGEN STORAGE OF PLANT CELLS AND TISSUES

(Cooling Curves)



a. Fast cooling: direct immersion in liquid nitrogen.

b. Slow cooling: controlled freezing

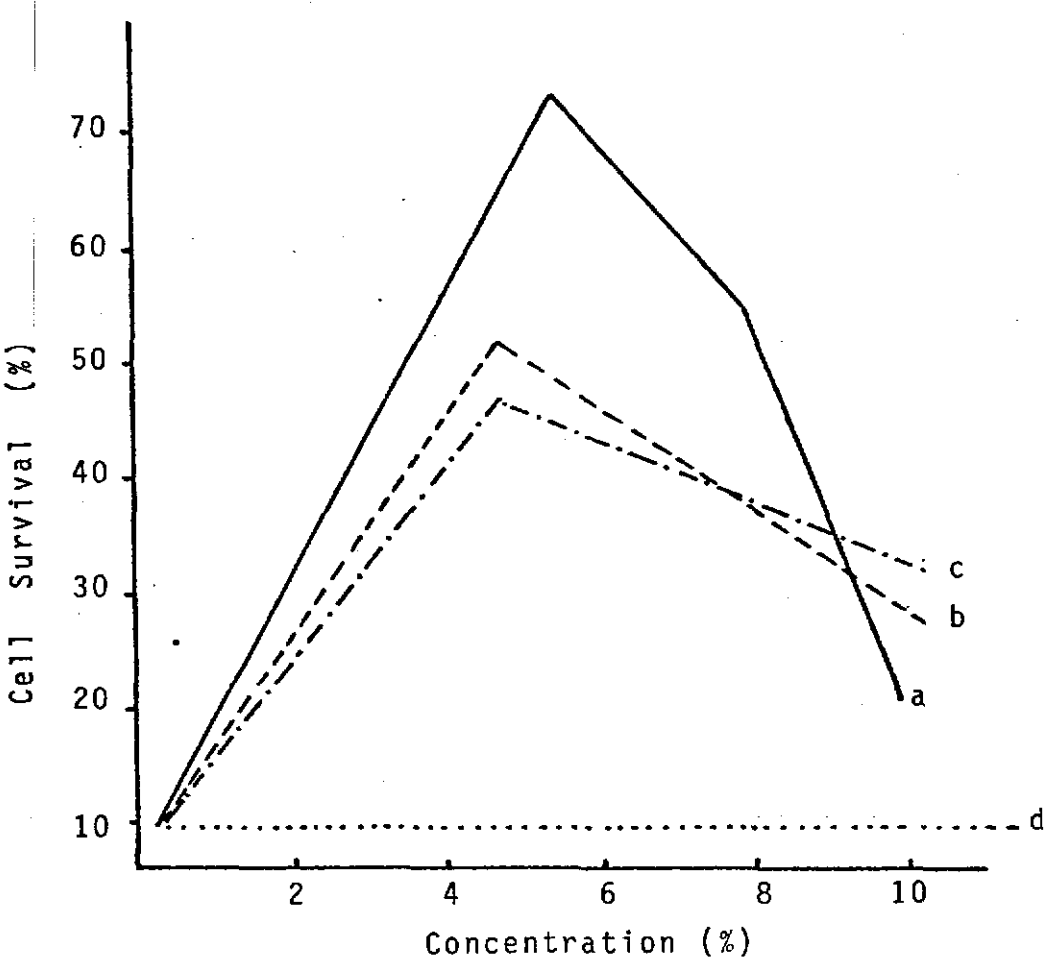
c. Fast warming: liquid nitrogen to 37°C (after Seibert and Wetherbee, 1977) (64)

FIG. 11

FIG. 12

LIQUID NITROGEN STORAGE OF PLANT CELLS

(Effect of cryoprotectants)



a. DMSO
b. DMSO + glycerol
c. Glycerol
d. Sucrose

(after Nag & Street, 1973) (60)

from liquid nitrogen, it is imperative in practice that plants can be recovered from these cells. Aside from the work with carrot (60) there are almost no other examples to imply that this occurs widely. Therefore, to utilize liquid nitrogen storage in large germplasm conservation programs, cells and callus cultures are not recommended; instead, meristems and shoot-tip should be used.

After the initial successful work on the cryopreservation of hardy winter vegetative buds (65) down to -50°C, research began for its use in other non-hardy species.

Relative success on liquid nitrogen preservation of carnation shoot apices (66) was achieved by very fast cooling rates (1000°C/minute) and the use of cryoprotectants. Likewise, potato apices of one variety regenerated shoots in 28 out of 188 frozen cultures (67). In both cases, only the meristematic cells survived, the leaf primordia being damaged.

On the other hand, recent work with pea meristems (38) showed higher survival rates (60-70%) after four months storage in liquid nitrogen; in this case very slow cooling rates (0.5 - 0.6°C/minute) were used.

High levels of survival, as well as rapid plant regeneration after thawing are desirable in order to prevent the possibilities of cell selection due to freezing. These attributes will also secure the utilization of the technique in a dependable fashion.

b. Serial transfer of cultures:

Tissue cultures could be simply maintained by frequent passages into fresh media. This technique however, requires intensive labor and incurs the risk of microbial contamination, equipment failure and, on a long-term basis, possible chromosomal aberrations and loss of organogenic potential (64). The use of meristem cultures should avoid the risks of cytogenetic and organogenic changes, but still become a high labor intensive operation.

c. Storage under minimal growth conditions:

A practical and highly dependable storage technique, using meristem cultures, would consist in maintaining the cultures at such a low rate of growth that the transfer interval would be extended to years. This system should secure high genotype stability since the meristems would keep growing (i.e. the meristems will keep their dinamic organization), but at a low rate. Retrieval of cultures from storage and multiplication of plants for field planting could also be readily done.

Work was done with potatoes (68) on the storage of shoot-tips under 6°C and with the addition of growth retarding substances to the culture media. Biochemically (i.e. electrophoretic protein patterns), the retrieved cultures maintained their clonal stability. Similarly, strawberry meristem plantlets were stored at 4°C for up to 6 years (69) without apparent damage.

It is unlikely that serious aberrations (eg. mutations) may occur in those cultures; however transcient, physiological, changes such as cold acclimation may happen in some materials. Such variations should revert back to normal following short periods of field planting.

Gradual dehydration of the cultures during storage may be a drawback of this technique. The use of closures with low water permeability should reduce dehydration. Substantial or total reduction of water loss by evaporation from the agar medium can be achieved by mineral oil overlay of the cultures. Under such conditions, oxygen seems not to limit the survival of the cultures, even though the growth rate is reduced (70).

III. CONSERVATION OF CASSAVA GERMPLASM BY MEANS OF MERISTEM CULTURE

Conventional maintenance of the cassava germplasm collection is by continuous vegetative field cultivation. Often, new cassava plantings are made directly from freshly cut sticks (stem cuttings) of old plantings.

Short-term stored cuttings are often prone to dehydration, microbial or insect attack and premature sprouting (71), all produce poor plant vigor. Cuttings treated with fungicide-insecticide mixtures gave high sprouting rates after 90 days storage at room temperature (71). Although long-term (i.e. years) storage of cassava by means of stem cuttings is quite unlike, chemical inhibition of sprouting may be suitable for short-term storage. Furthermore, stem cuttings, because of their large size, would probably carry up systemic contaminants from one generation to the next.

Recent work carried out on the cryopreservation of cassava has shown that the meristems are very sensitive to various cryoprotectants and that these substances apparently impair the organogenic expression of the meristem after thawing; lower concentrations of the cryoprotectants are inadequate to prevent freezing damage of the meristems (38).

Thus, there is still more work ahead to develop cryopreservation methods of cassava meristems to the degree that it could be utilized on a large germplasm preservation program. Alternatively, the growth rate of cassava meristem cultures can be modulated in such a way that growth rates as low as 1-2 mm/month can be achieved (72) through culture under minimal growth conditions.

a. Minimum growth storage of cassava meristem cultures:

From results of our research (72) it has emerged that the rate of growth of node-bud cuttings (see Section E) maintained at 20°C can be reduced to one tenth of the cultures grown at 30°C day and 25°C night temperatures (see Table 4). The growth could be further reduced, without significant loss in survival, if the sucrose concentration of the medium increases up to 4-5%; however, higher sucrose levels were detrimental to the cultures. The addition of BAP to the medium, further slows down the growth rate. Cultures that were maintained at 15°C became gradually chlorotic and senescent after 1-2 month storage.

Cassava meristem cultures have been stored at these conditions for up to 14 months and still are able to regenerate normal plants.

Cytokinins on the one hand and high sucrose on the other, each tend to elicit axillary shoot growth, after protracted storage; even if the leaves have senesced. Indeed, leaf senescence per se seems to lead to axillary shoot growth.

Trials have been initiated to store cassava as multiple shoot cultures. In this form, potentially more materials can be maintained at any one time per unit of

TABLE 4

EFFECTS OF TEMPERATURE, SUCROSE AND CYTOKININS ON THE GROWTH (cm/month) OF CASSAVA SHOOT TIP CULTURES

Media	% Sucrose	M. Brasil 12		M. Col 33	
		30°C/25°C[2]	20°C/20°C	30°C/25°C	20°C/20°C
Basal[1]	2	5.5	0.5	5.2	0.5
	3	3.5	0.4	3.4	1.2
	4	6.2	0.3	5.8	0.2
	6	3.8	0.2*	4.6	0.1*
	8	0.8*	0.05**	0.5*	0.07*
Basal + 0.01 mg/l BAP	2	2.8	0.3	2.5	0.4
	3	3.0	0.4	3.2	0.5
	4	1.1	0.2	0.7	0.1
	6	1.2	0.2	0.6	0.08*
	8	0.7*	0.05*	0.4*	0.06**

Figures represent average of four replicates.

1. Basal medium: MS + 0.1 mg/l NAA + 0.1 mg/l GA.
2. Day/night temperature regime.

Survival after 4 months storage: ** = 10%; * = 60%; all others = 100%

shelf area and the propagation of materials will be speeded up.

Wherever required, rapid recovery of plants from storage under minimum growth conditions can be done by means of the node-bud cutting technique.

Finally, through this method we intend to store nearly 3,000 cassava accessions in a 4 x 5 x 2.5 room with a shelf area of about 35 m^2.

b. Technique:

1. To store multiple shoot cultures, first of all follow procedures described in Section E for the induction of rosette cultures.

2. Transfer rosette cultures to the following storage media:

   MS (4% sucrose) + 0.05 mg/l BAP; or
   MS (1% sucrose) + 0.1 mg/l BAP

   Agar: 0.8%

   Use large (150 x 25 mm) test tubes

   To prepare the media see Sections C and E.

   In order to store single shoot cultures, plant terminal and axillary node-bud cuttings in the following media:

   MS (4% sucrose) + 0.02 mg/l BAP; or
   MS (1% sucrose) + 0.05 mg/l BAP

   Agar: 0.8%

Use large (150 x 25 mm) test tubes

3. Incubate the cultures under the conditions described in Section A.

4. After about 2 weeks, the rosette cultures begin to proliferate axillary shoots. The single shoot cultures initiate rooting.

5. Prior to transfer to the storage conditions, evaluate the cultures: average size and number of shoots per culture, number of nodes per shoot, rooting, callusing, etc. Green shoots, with little or no basal callus and with small roots whould be preferred for storage.

6. Place the cultures under storage conditions. Five-ten tubes per accession could be stored, depending on space availability. Storage conditions: 20°C day and night, 12 hour photoperiod, 2,000 lux illumination.

7. Monthly observation of growth can be carried out to monitor the changes during storage.

8. Every 6 months retrieve a sample of each accession and recover plants for field trials:

   -- Cut terminal or axillary node-bud cuttings and plant in the rooting medium: 1/2 MS + 4% sucrose; agar: 0.8%.

   -- Incubate cultures for about one week, as at 3 above.

   -- Harden the cultures as in Section A.

-- Potting as in Section A.

-- Field planting as in Section A.

SECTION H

CASSAVA MERISTEM CULTURE: SUMMARY

Cassava is a major source of calories for a large portion of the world's population. Both the tuberous roots and the leaves can also be used as livestock feed; though the tubers are low in proteins, the foliage is quite high. Recent trends in the exploitation of the crop in certain areas of the world focus on the potential of cassava for use in industry and as an attractive alternative to other energy sources.

However, because cassava is preferentially propagated by vegetative means it is often exposed to a wide range of pests and diseases, being the systemic ones more difficult to detect and control. The pest and disease risk is compounded by the fact that due to its long growing cycle and very low multiplication by conventional procedures, the crop's germplasm resources are in danger of irreperable losses.

Conventional maintenance of large cassava germplasm collections is done by continuous vegetative field cultivation: this is an intensive and very expensive labor. Similarly, because of the real risks to disseminate pests and diseases, various regions of the world have erected strict quarantine regulations which prevent the introduction of cassava vegetative materials.

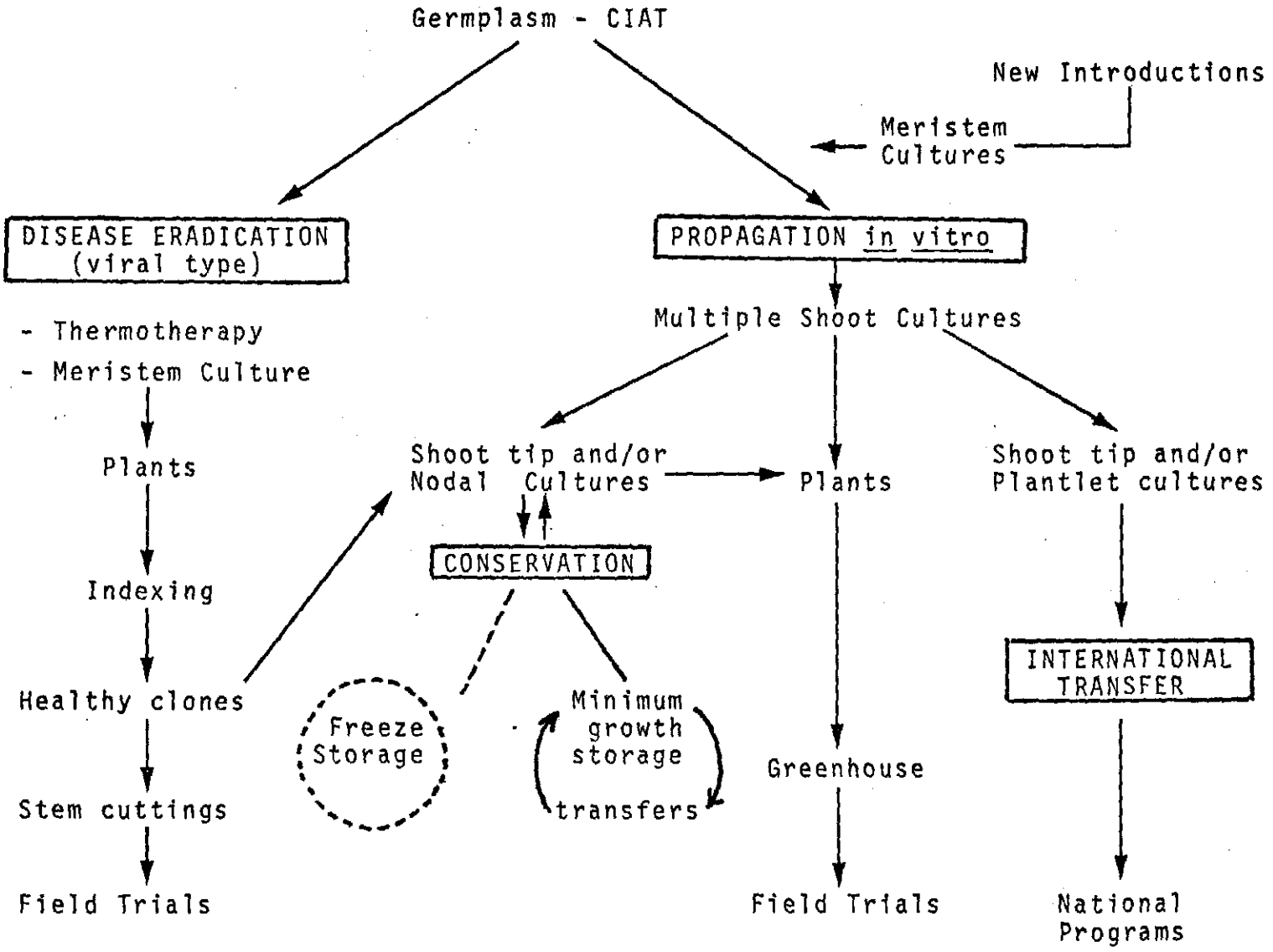
Meristem culture methods, due to their potentially high propagation rates, coupled with their freedom from microorganisms, small space requirements, high genotype stability and relatively simple handling procedures, can be used for the

maintenance and international exchange of cassava germplasm. Fig. 13 depicts the various steps in the application of meristem culture in cassava.

These tissue culture methods can therefore be used very profitably to back up current, more conventional, procedures of cassava propagation, germplasm conservation and distribution.

Work carried out in CIAT during the last year and a half, along these lines, has shown that this is quite feasible. Further work integrated with other areas of cassava research will, in a short-term basis, provide practical procedures to handle cassava germplasm resources in such a way as to guarantee its safe conservation and transfer.

FIG. 13 OUTLINE OF THE VARIOUS STEPS IN THE APPLICATION OF MERISTEM CULTURE TO CASSAVA



SECTION I

OTHER CASSAVA TISSUE CULTURE SYSTEMS

As discussed earlier (Section G), cell, callus and anther cultures are prone to cytogenetic instabilities, during culture. Both the tissue utilized as initial explant and the composition of the culture media, influence the occurrence of variant cells. These tissue culture systems are thus not recommended for a germplasm conservation program. However, their innate capacity to change could be coupled to the use of stress agents (chemical or physical), in order to produce cell variants or even mutant lines.

Various applications of these systems to cassava can be envisaged: large populations of cells or protoplasts could be subjected to different stresses (eg. pathotoxins, amino acid analogs, high osmotic concentration, high aluminum, etc.) and select resistant cell lines. The use of haploid tissues would greatly facilitate the task of mutation induction, selection and stabilization. On the other hand, protoplast fusion could allow to overcome inter or intra-genetic incompatibility barriers. Anther and pollen culture could serve to produce doubled haploids for use in breeding.

Specific breeding problems such as cyanide production in hybrid lines could be approached in tissue culture to look for systems which could break somewhere the biosynthetic pathway and yield clones with very low or nil cyanide.

In order to at least see the potential use of those methods, it is of paramount importance that procedures be developed which can allow to regenerate complete plants from cell

and callus cultures in cassava.

Some work has been done elsewhere (15, 73, 74, 75) to grow callus cultures from stem, root tubers and anthers; yet no shoot organogenesis has been achieved in any of these. Cassava, like other tuber crops, seems to fall into the recalcitrant category as far as shoot regeneration from callus is concerned. Recent work done with protoplast culture* looks quite promising since sporadic shoot initiation was observed from protoplast calli. Further improvement of the protoplast culture technique to consistently regenerate plants would open tremendous possibilities in the applications to cassava.

We have studied the ability of various tissues of cassava to form callus. Shoot tips formed larger and more homogenous calli than leaf, stem and petiole explants. Leaf mesophyll explants also gave good callus next after shoot tips. The MS basal medium supplement with 3% sucrose and 4 mg/l 2, 4-D or NAA and 0.2 mg/l 2iP or 0.5 mg/l BAP was better for leaf explants; lower auxin (2 mg/l), and NAA better than 2, 4-D, was required for callus growth from shoot tips. Upon transfer of callus masses derived from shoot tip explants to liquid rotary media, greenish cell suspensions were produced. For cell suspension culture, the MS basal medium was supplemented with 2% sucrose, 0.05 mg/l BAP, 0.05 mg/l GA and 0.02 mg/l NAA.

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* Recent personal communication from Dr. J. Shepard.

SECTION J

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7. Plant Tissue Culture Abstracts. Published by GIBCO, four issues a year.

SECTION K

LIST OF MAIN SUPPLIERS

1. Inorganic constituents of culture media:

   -- Fisher Scientific Co.
   711 Forbes Ave.
   Pittsburg, PA 15219
   U.S.A.

   -- Mallinckrodt
   3016 East 44th Street
   P.O. Box 58645
   Los Angeles, CA 90058
   U.S.A.

2. Pre-made MS salt mixture (No. M11-1117):

   -- GIBCO
   3171 Staley Road
   Grand Island, NY 14072
   U.S.A.

3. Organic constituents of culture media, including hormones and vitamins:

   -- Sigma Chemical Co.
   P.O. Box 14508
   St. Louis, Missouri 63178
   U.S.A.

4. Laminar Flow Transfer Cabinets:

   -- ENVIRCO
   620 Supertest Road
   Downview, Ontario M3J 2M8
   CANADA

Laminar Flow Transfer Cabinets (con't):

-- Environmental Air Control, Inc.
747 Bowman Ave.
Hagerstown, Maryland 21740
U.S.A.

5. Gyratory Shakers:

-- New Brunswick Scientific Co., Inc.
P.O. Box 31
Burlington, Ontario L7R 3X9
CANADA

6. Environmental Growth Chambers (CONVIRON):

-- Controlled Environments
1461 James Street
Winnipeg, Manitoba R3H OW9
CANADA

7. Illumination devises: lamps, ballasts, etc.:

-- GTE Sylvania, Lighting Products Group
100 Endicott Street
Danvers, Mass. 01923
U.S.A.

8. Sterilizing Units:

-- Millipore Corporation
P.O. Box 680
New York, NY 10010
U.S.A.

9. Test Tube racks:

-- Fisher Scientific Co.
(Same address as No. 1)

10. 45°Slant Tissue Culture Tube Racks:

-- Limited Plastics
P.O. Box 89
Lemoncove, CA 93244
U.S.A.

11. General Glassware: culture tubes (pyrex), culture tubes (disposable), culture tube closures (Morton, Bacti-cappall), beakers, erlenmeyer flasks, pippets, etc.

-- Scientific Products
17111 Red Hill Ave.
Irvine, CA 92705
U.S.A.

-- Kimble
Owens, Illinois
P.O. Box 1035
U.S.A.

-- American International Container, Inc.
3724 N.W. 73rd Street
Miami, Florida 33147
U.S.A.

12. General Plasticware:

-- Kimble
(Same address as No. 11)

13. Disposable pipettes, Drummond:

-- Drummond Scientific Co.
Broomall, Pennsylvania
U.S.A.

14. Anti-Microbial protection devises (masks, S-entries, surface sterilizants, etc.)

-- Arbrook, Inc.
P.O. Box 130
Arlington, Texas 76010
U.S.A.

15. Plant Product Fertilizers:

-- Plant Products Co., Ltd.
Bramalea, Ontario L6 1G1
CANADA

16. Surgical supplies: scalpels, twizers, needles, etc.

-- Fisher Scientific Co.
(Same address as No. 1)

17. Other electronic equipment: pH meters, Thermocouples, pumps, recorders, hygrothermographs, etc.

-- Cole-Permer Instruments Co.
7425 North Oak Park Ave.
Chicago, Illinois 60648
U.S.A.