PRACTICAL MANUALS FOR HANDLING CROP GERMPLASM *IN VITRO* 2

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D.R. VUYLSTEKE

INTERNATIONAL BOARD FOR PLANT GENETIC RESOURCES

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FOREWORD

In vitro methods are assuming an increasingly important rôle in the conservation of plant genetic resources, particularly for clonally-propagated samples and for species which, for various reasons, are difficult to conserve as seed. Diverse crops fall into these categories and range from root and tuber crops such as sweet potato, yam and cassava, to many tropical tree fruits and several industrial crops such as rubber and cocoa.

The first volume in this series - Practical Manuals for Handling Crop Germplasm *In Vitro* - covered meristem-tip culture and virus indexing for sweet potato.

This volume, the second in the series, collates available technical data on *in vitro* methods for the propagation, conservation and distribution of *Musa* (banana and plantain) germplasm. The author is an expert in the *in vitro* handling of *Musa* and has drawn on the experiences and publications of other scientists to provide a comprehensive guide to current techniques. Practical information required to undertake the *in vitro* conservation of *Musa* is presented. The reader will be able to update the information as necessary through contacts provided here as literature citations and as entries in the IBPGR *In Vitro* Conservation Databases.

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1. INTRODUCTION

Bananas and plantains (*Musa* spp.) are a major staple food for many millions of people throughout the humid and subhumid tropics. World production is estimated at more than 62 million tonnes annually. The world export trade in bananas amounts to about seven million tonnes, indicating that the crop is mainly grown as a food crop for local consumption. The International Board for Plant Genetic Resources (IBPGR) has recognized the economical and social importance of bananas and plantains and considers the conservation of *Musa* germplasm as a high priority (IBPGR, 1981).

In the past decade, the most dramatic event affecting banana and plantain production has been the appearance and spread of more virulent forms of the Sigatoka leaf spot diseases. Black Sigatoka and Black Leaf Streak are now pantropic and have become the overriding constraints to expanding cultivation of edible *Musa*. The causal agent of Black Sigatoka, *Mycosphaerella fijiensis*, is a fungus which attacks the leaves, thereby reducing yields by 30-50% (Stover, 1983; INIBAP, 1987a). The appearance of races of the *Fusarium* wilt pathogen that attack cultivars previously considered to be resistant presents yet another threat to the crop.

These problems have prompted an increased international effort towards the genetic improvement of this important crop. Of paramount importance to these breeding endeavours is the selection, conservation and movement of germplasm. However, since cultivated *Musa is* notoriously sterile and thus must be vegetatively propagated, several difficulties arise:

- germplasm exchange is impeded by the lengthy periods in quarantine that conventional vegetative propagules must undergo due to their innate risk of disseminating pests and pathogens (Chiarappa and Karpati, 1984; Stover, 1977)
- germplasm conservation in field genebanks is at risk of disease and natural disasters, and requires a large input in space and labour (De Langhe, 1984)
- low rates of multiplication by conventional means hamper the rapid dissemination of selected, disease-resistant cultivars.

However, recent developments emphasize the potential that the techniques of plant tissue culture offer in the propagation, conservation and genetic improvement of *Musa* (Cronauer and Krikorian, 1986a; Krikorian and Cronauer, 1984a; Stover and Buddenhagen, 1986). Shoot-tip culture has already been applied successfully to the rapid clonal propagation of many different *Musa* species and cultivars and as a means of germplasm exchange and conservation. Although other *in vito* culture systems also hold great promise, this manual only deals with shoot-tip/meristem culture. This is the only method yet known by which plants have been regenerated in a consistent and routine manner. Moreover, it is the technique *par excellence* to minimize somaclonal variation, which is an important consideration when handling crop germplasm *in vitro* (Scowcroft, 1984).

2. BRIEF HISTORY OF SHOOT-TIP CULTURE OF *MUSA*

Rapid clonal propagation was the first major practical application of the science of plant tissue culture (George and Sherrington, 1984). Other important applications are the eradication of diseases and the conservation and exchange of germplasm (De Langhe, 1984; Withers and Williams, 1985).

These were also the objectives of the first applications of tissue culture to *Musa*. The earliest reports of banana plants produced by *in vitro* shoot-tip culture came from Taiwan, China, in the early 1970s (Ma and Shii, 1972; 1974; Ma *et al.*, 1978). Berg and Bustamante (1974) used meristem culture combined with heat therapy, to produce virus-free bananas in Honduras. A team at the University of the Philippines produced banana shoots *in vitro* for mutation induction by irradiation (De Guzman *et al.*, 1976; 1980). These first reports were based on research carried out on a very limited number of dessert banana cultivars of the *Musa* AAA group, mainly Cavendish types.

Since 1980, however, a wide range of *Musa* species and cultivars of all genomic constitutions have been found amenable to *in vitro* shoot-tip culture (Cronauer and Krikorian, 1984a; 1984b; Jarret *et al.*, 1985; Müller and Sandoval, 1986; Novak *et al.*, 1986; Sun, 1985; Vessey and Rivera, 1981; Vuylsteke, 1983; Vuylsteke and De Langhe, 1985; Wong, 1986; Zamora *et al.*, 1986). *In vitro* multiplication rates are several orders of magnitude higher than in conventional propagation. Plant production *in vitro* is then only limited by the number of skilled technicians who can handle large numbers of shoot-tip cultures aseptically. Large-scale field establishment of *in vitro* propagated plants of banana and plantain has been reported in Taiwan, China (Hwang *et al.*,1984), the Philippines (Epp,1987; Zamora *et al.*, 1986), Australia (Johns, 1985), Costa Rica (Jarret *et al.*, 1985), Jamaica (Oglesby and Griffis, 1986; Stover, 1987), Puerto Rico (Pool and Irizarry, 1987), US Virgin Islands (Ramcharan *et al.*, 1987), Colombia (R. Swennen, pers. comm., 1987), Israel (Reuveni *et al.*, 1985), Morocco (Kenny and Aaouine, 1987), Cameroun (C. Teisson, pers. comm., 1987) and Nigeria (IITA, 1986; Vuylsteke *et al.*, 1988). In the field, growth of *in vitro* propagated plants is vigorous due to the disease-free nature of the planting material (Hwang *et al.*, 1984; Hwang and Ko, 1987; O. Reuveni, pers. comm., 1987).

Musa germplasm in tissue culture form has been exchanged internationally since the first half of this decade (Jarret *et al.*, 1985; Zamora *et al.*, 1986). Since 1985, the International Institute of Tropical Agriculture (IITA) at Ibadan, Nigeria, has successfully used meristem cultures for interregional and intercontinental exchange of germplasm; more than 120 *Musa* species and cultivars have been introduced and over 100 distributed to many parts of the world (IITA, 1988).

There is also increasing interest in the application of *in vitro* approaches to the conservation of *Musa* germplasm. Satisfactory results have been obtained using shoot-tip cultures stored under minimal growth conditions (Banerjee and De Langhe, 1985; Jarret *et al.*, 1986; Zamora *et al.*, 1987).

This short overview of developments during the past 15 years emphasizes the potential value of aseptic *in vitro* culture techniques as tools in the handling of *Musa* germplasm. Because shoot-tip culture is a relatively simple technique by which numerous different types of bananas and plantains have been propagated on defined culture media, it is recognized as a satisfactory and reproducible procedure for handling *Musa* germplasm *in vitro*.

In addition to the procedures and literature cited in this manual, further information on the application of *in vitro* techniques to *Musa* can be obtained from the IBPGR *In Vitro* Conservation Databases (Wheelans and Withers, 1984). A summary of relevant entries in the Databases is given in Appendix II.

3. MATERIALS AND EQUIPMENT

Successful *in vitro* culture results from the interplay of the plant material, the medium in use, and the culture environment. These factors, are outlined below; procedures involved in the shoot-tip culture of *Musa*, are covered in the following section (4: *In vitro* propagation methods).

3.1 Laboratory facilities

Ideally, a plant tissue culture facility should consist of separate rooms providing a media preparation area, a transfer area for aseptic manipulations, and a culture incubation area. When this is not possible, the transfer area can be set up in an infrequently used area of the main laboratory. Essential services such as electricity and water should be available.

3.1.1 *Media preparation area*

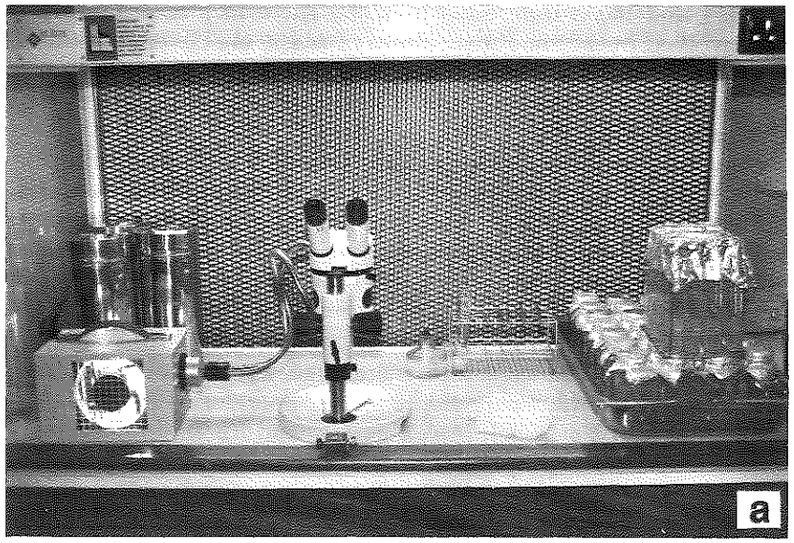
This should have ample bench and storage space for chemicals, glassware and culture vessels. Bench surfaces should be smooth, preferably covered by an easily cleanable material (e.g. 'Formica'). A refrigerator and freezer are necessary for the storage of stock solutions, prepared media and some chemicals. Other essential equipment includes a pH meter, a top-loading balance (preferably with a precision of 1 mg or less and a weighing range of 0-200 g), a hotplate/stirrer, heating mantles and an adjustable volume dispenser or other suitable device to dispense aliquots of hot medium. An autoclave or domestic pressure cooker is required for sterilizing media, culture containers and dissecting instruments.

Glassware should include an assortment of beakers, graduated cylinders, large boiling flasks, volumetric flasks, Erlenmeyer flasks, pipettes and storage bottles. Culture vessels may be of glass or dispensable material (see 3.4.1: Culture vessels). Hot and cold water supplies, and one or more sinks are required for washing used glassware. An automatic washing machine can handle much of the routine washing. A source of distilled or deionized and distilled water is essential, since tap water is unsuitable for plant tissue culture media. The most common and preferred method of water purification for tissue culture use is deionization followed by distillation.

3.1.2 *Transfer area*

The need for asepsis is a critical factor to consider when setting up the culture facilities. Aseptic work without a transfer cabinet or laminar air-flow cabinet is difficult in the humid tropical regions where bananas thrive. The atmosphere of forests and plantations carries high densities of micro-organisms and their spores, making the maintenance of asepsis a serious practical problem (Sossou *et al.*, 1987). The most desirable arrangement is a separate, dust-free room equipped with one or more laminar air-flow cabinets and possibly fitted with ultraviolet light. Simpler transfer cabinets without filtered air, can consist of an enclosed plastic box or a wooden hood. These may be adequate if they are installed in an isolated room in which air movement is kept to a minimum and if all surfaces can be thoroughly cleaned and disinfected.

To reduce the risk of contamination, a high level of cleanliness has to be maintained and it is preferable that the transfer room only be used for the aseptic manipulation of *in vitro* cultures.



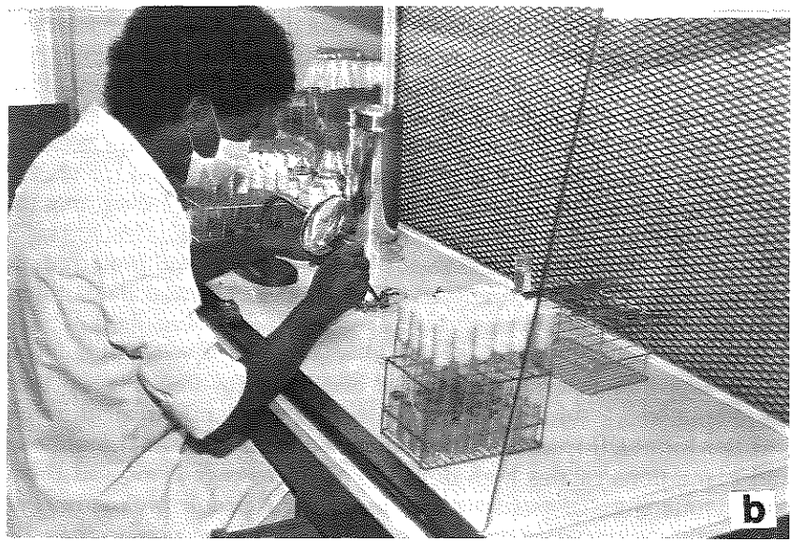


Figure 1. Preparation of the laminar air-flow cabinet for aseptic manipulations.: (a) Arrangement with stereo microscope. From left to right: sterilizing boxes containing sterile Petri dishes, light source, stereo microscope, alcohol lamp, ethanol dip with dissecting instruments on a test tube rack, medium in test tubes and glass jars; (b) arrangement with magnifier.

Equipment needed includes an assortment of dissecting instruments (scalpels with removable blades, and forceps), an ethanol dip, an alcohol lamp or bunsen burner, a spray bottle of ethanol, and a stereo microscope with light source (Fig. 1a). The latter can be substituted for by a magnifier mounted on a flexible arm (Fig.1b). In some laboratories, shoot-tip dissection is performed using a headband binocular magnifier (M. Bustamante, pers. comm., 1983). Except for the dissection of shoot-tips, aseptic manipulations do not generally require a magnifying aid.

3.1.3 Culture incubation area

A growth chamber or a windowless room with environmental control is preferred. However, complete control is not absolutely necessary, as bananas and plantains can be cultured under a relatively wide range of environmental conditions. An air conditioning unit is suitable for temperature control.

Cultures are normally kept in racks on stacks of shelves illuminated by banks of fluorescent lamps. Artificial light is usually provided by cool-white fluorescent tubes of 40 W. The ballasts in the fluorescent lights are often installed outside the culture room as they generate heat. The photoperiod is controlled by means of time switches installed in each culture room or even on each set of shelves. For maximum use of space, shelves can be mounted on wheels so they can be placed side by side. At IITA, a culture room of 10 m^2 can, in this way, hold 15 000 - 30 000 cultures depending on the type of culture vessel used. When using liquid cultures, a gyratory shaker is necessary to ensure adequate aeration. Further details of the environmental conditions in the culture room are given elsewhere (see 3.4: Culture environment). Further reading on laboratory facilities is available in Biondi and Thorpe (1981) and Withers (1985).

3.2 Plant material

Shoot-tips can be obtained from all plant parts that contain a shoot meristem. Growth response and explant survival in culture do not differ among shoot apices obtained from the parental pseudostem, its suckers, peepers, lateral buds or even very small eyes (Jarret *et al.*, 1985; Vuylsteke and De Langhe, 1985). Apparently, the physiological and ontogenetic age of the shoot-tip does not influence the behaviour of the explant in culture. Nor are there any reports on the effect on culture performance of the season in which the explants are obtained. However, buds, peepers and small sword suckers (Fig. 2) are the preferred source material due to their greater ease of handling (Jarret *et al.*, 1985) and because there is less damage to the parent stool during their removal.

Explant material should only be collected from flowering plants, to ascertain trueness-to-type. Source plants should also be free of disease and growing vigorously. Special pretreatments of the mother plant are not required. Terminal inflorescence apices and young flower buds in bract axils are capable of producing plants using *in vitro* protocols similar to the shoot-tip culture technique (Bakry *et al.*, 1985; Cronauer and Krikorian, 1985, 1986b; Ma *et al.*, 1978). Cronauer and Krikorian (1986b) report also having achieved this in False Horn plantains, which do not have a terminal apex, but rather terminate with a floral structure. (For more

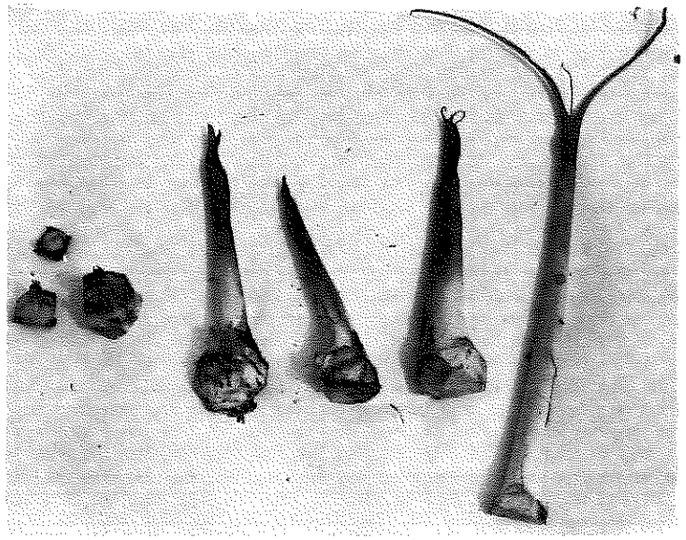


Figure 2. Preferred plant material for the initiation of *Musa* shoot-tip cultures: Left, buds; right, peepers and small sword suckers.

information of the specific taxonomy of plantains (*Musa* AAB), refer to Tezanas du Montcel *et al.*, 1983). The terminal floral apex and the axillary flower buds manifest morphogenic plasticity in their embryonic stage and can be induced to revert to vegetative meristems, multiplying *in vitro*.

A wide range of species and cultivars, representing the major part of the genetic variability within the genus *Musa*, have been successfully propagated *in vitro*. At IITA, the author has micropropagated 180 different types of wild and cultivated *Musa*: 15 wild *Musa acuminata* (several subspecies), 4 wild *Musa balbisiana*, 13 AA and 2 AB clones, 9 AAA and 6 AAB dessert bananas, 6 East African AAA (beer and cooking bananas), 1 AAAA, 102 AAB plantains, 21 ABB cooking bananas, and *Musa textilis* (Appendix 1). (The genomic constitution and ploidy of cultivated *Musa* in the section Eumusa are designated by the relative contributions of the two wild species *Musa acuminata* (A genome) and *Musa balbisiana* (B genome), based on a diagnostic scoring method (see Simmonds and Shepherd, 1955; Stover and Simmonds, 1987)).

C. Teisson (pers. comm., 1988) has also tested AAAB and ABBB bananas. Zamora *et al.* (1986) maintained *in vitro* 91 accessions from Malaysia, the Philippines, and Thailand. Sun (1985) reported the successful *in vitro* multiplication of 93 species and cultivars of six genomic groups. Jarret *et al.* (1985), Vuylsteke and De Langhe (1985), Novak *et al.* (1986) and Wong (1986) also describe successful *in vitro* culture and plant production in cultivars belonging to four to six different genomic groups.

3.3 Culture media

3.3.1 *Media composition*

Successful *in vitro* culture depends largely on the choice of nutrient medium, including its chemical composition and physical form (Murashige, 1974). Medium containing the Murashige and Skoog (MS) mineral salt mixture (Murashige and Skoog, 1962; see Table 1) is very suitable for banana and plantain shoot-tip culture. It is the most widely used medium. Some authors (Epp, 1987; Hwang *et al.* , 1984; Ma and Shii, 1972) report the use of Smith and Murashige (1970), medium in which the salt composition is identical to that of MS except for additional phosphate. C. Teisson (pers. comm., 1988) also uses an increased phosphate concentration (200 mg l^{-1} KH_2PO_4). However, most investigators use slightly modified MS medium, with alterations only to the organic constituents (carbon, vitamin, amino acid and growth regulator supplement; see Table 2). As a guide, one such effective modified MS medium is given in Table 3. Other suitable media formulations based on the MS salt mixture are available in some of the references cited below.

Sucrose is the preferred carbon source and is used at a concentration of 2-4% (w/v), although 3% is most common. Sucrose can be substituted by dextrose at the same concentration (De Guzman *et al.*, 1980; Krikorian and Cronauer, 1984b). Sucrose of analytical grade is expensive and can be substituted by refined grocery sugar. The sugar alcohol myo-inositol (100 mg l^{-1}) is not essential, but is routinely added in many laboratories. Similarly, the amino acid glycine is not essential but it provides a source of nitrogen that is immediately available to cultured tissues (George and Sherrington, 1984). Adding 15% (v/v) coconut water may be beneficial in inducing growth in banana shoot-tips (Krikorian and Cronauer, 1984b). However, inclusion of organic additives such as coconut water or casein hydrolysate is not absolutely necessary and is undesirable, as it results in the use of chemically undefined media. Of the vitamins, only thiamine may be necessary (Krikorian and Cronauer, 1984b). Thiamine is added at 0.4-1.0 mg l^{-1}. Nicotinic acid and pyridoxine (both at 0.5 mg l^{-1}) are frequently included. Other vitamin mixtures, such as those of Nitsch (De Guzman *et al.*, 1980) and Morel (C. Teisson, pers. comm. 1988), have also been used.

MS medium has also been supplemented with reducing agents (antioxidants) such as ascorbic acid (10-100 mg l^{-1}), citric acid (150 mg l^{-1}) and L-cysteine (2-40 mg l^{-1}) (Gupta, 1986; Mante and Tepper, 1983; F. Novak, pers. comm., 1988; Vuylsteke and De Langhe, 1985) to reduce blackening of shoot-tips (also see 4.1: Culture initiation; and 7.1: Blackening of shoot-tips).

Plant growth regulators (sometimes erroneously called hormones) are essential for the manipulation of growth and development of explants *in vitro*. Their concentration and ratio in the medium often determine the pattern of development in culture. For *Musa* shoot-tip culture, only cytokinins and auxins are required. The most commonly used auxins are IAA (indole-3-acetic acid), NAA (α-naphthalene-acetic acid) and IBA (indole-3-butyric acid). 6-benzylaminopurine (BAP) is the cytokinin of choice for the induction of shoot bud proliferation *in vitro*. BAP has been found to be superior to kinetin (Cronauer and Krikorian, 1984b; Damasco and Barba, 1985; Wong, 1986; Zamora *et al.*, 1986), isopentyladenine (2iP; Dore Swamy *et al.*, 1983) and zeatin (Vuylsteke and De Langhe, 1985). Concentrations of growth regulators are discussed below (see 4: *In vitro* propagation methods).

Table 1. Composition of the Murashige and Skoog (1962) mineral salt mixture

Major salts		Minor salts	
Salt	mg l^{-1}	Salt	mg l^{-1}
KNO_3	1900	$MnSO_4.4H_2O$[1]	22.3
NH_4NO_3	1650	H_3BO_3	6.2
$CaCl_2.2H_2O$	440	$ZnSO_4.4H_2O$	8.6
$MgSO_4.7H_2O$	370	KI	0.83
KH_2PO_4	170	$Na_2MoO_4.2H_2O$	0.25
$FeSO_4.7H_2O$	27.8	$CuSO_4.5H_2O$	0.025
$Na_2EDTA.2H_2O$	37.3	$CoCl_2.6H_2O$	0.025

1. The author prepares MS medium with monohydrated $MnSO_4.H_2O$ at 16.9 mg l^{-1}, i.e. the same Mn^{2+} concentration of 0.1 mM.

Table 2. Organic supplements: range of concentrations found in media for *Musa* shoot-tip culture

Compound	mg l^{-1}
Sucrose	20 000 - 40 000
Myo-inositol	0 - 100
Glycine	0 - 2
Thiamine	0.4 - 1
Pyridoxine	0 - 0.5
Nicotinic acid	0 - 0.5
Ascorbic acid	0 - 100
Indole-3-acetic acid (IAA)	0 - 0.2
α -naphthaleneacetic acid (NAA)	0 - 0.2
6-benzylaminopurine (BAP)	0.2 - 10
Agar	4500 - 8000

Adenine sulphate (160 mg l^{-1}) was added to MS medium by Ma and Shii (1972) and Hwang *et al.* (1984). This may be conducive to shoot initiation when kinetin is the sole cytokinin used. However, in the presence of BAP, the author did not observe a significant effect of adenine sulphate on shoot bud proliferation in cultures of Eumusa cultivars. Conversely, Mante and Tepper (1983) noted a synergistic interaction between BAP and adenine sulphate in promoting shoot development in *Musa textilis* cultures. The inclusion of activated charcoal is discussed below (see 4.3: Regeneration of plants).

Table 3. Composition and amount of stock solutions used in the preparation of media for *Musa* shoot-tip culture (modified MS medium as used at IITA)[1]

Stock solution	Ingredient	Amount (mg)	ml of stock solution per litre of medium multiplication	regeneration	hardening
A: Macro 1[2]	KNO_3	95000	20	20	10
	NH_4NO_3	82500			
	$CaCl_2 . 2H_2O$	22000			
B: Macro 2[2]	$MgSO_4 . 7H_2O$	18500	20	20	10
C: Macro 3[2]	KH_2PO_4	8500	20	20	10
D: Micro[3]	$MnSO_4 . H_2O$	1690	1	1	1
	H_3BO_3	620			
	KI	83			
	$ZnSO_4 .4H_2O$	860			
	$Na_2Mo_4 . 2H_2O$	25			
	$CuSO_4 . 5H_2O$	2.5			
	$CoCl_2 . 6H_2O$	2.5			
E: Fe-stock[4]	$FeSO_4 . 7H_2O$	2785	5	5	5
	$Na_2EDTA.2H_2O$	3725			
F: Vitamins and amino acids[3]	Glycine	200	1	1	1
	Thiamine.HCl	40			
	Pyridoxin.HCl	50			
	Nicotinic acid	50			
G: Antioxidant[5]	Ascorbic acid	1000	1	1	1
H: IAA[6]	IAA	8.75	1	0	0
I: NAA[6]	NAA	9.30	0	1	0
J: BAP[7]	BAP	11.25	20[8]	1	0

1. All media contain 30 g l^{-1} sucrose and are solidified with 5 g l^{-1} agar.
2. Dissolve in distilled water and bring final volume to 1000 ml.
3. Dissolve in distilled water and bring final volume to 100 ml.
4. Dissolve in distilled water by heating to boiling point and bring final volume to 500 ml.
5. Dissolve in distilled water and bring final volume to 50 ml.
6. Dissolve in a few drops of ethanol (95%) and bring final volume to 50 ml with distilled water.
7. Dissolve in 1 ml 1N NaOH and bring final volume to 50 ml with distilled water.
8. For multiplication medium, it is better to use a stock solution prepared by dissolving 112.5 mg BAP in 5 ml 1N NaOH and bringing the final volume to 50 ml with distilled water; use 2 ml to prepare 1 litre of multiplication medium.

Important aspects of the culture medium are its solid or liquid state and the pH. Semi-solid medium is more commonly used than liquid medium. In some laboratories, liquid and semi-solid media are alternated, because the former increased the growth rate and shoot proliferation (Cronauer and Krikorian, 1984b; Novak *et al.*, 1986). However, liquid cultures require expensive apparatus (gyratory shakers) and occupy more space in the culture room. Agar is widely used to solidify media. Its concentration ranges from 4.5 to 8.0 g l^{-1}. Because agar of tissue culture grade is costly, the lower concentration range of 5.0-6.0 g l^{-1} is generally recommended, as this still gives ample firmness to the medium. 'Gelrite' is an alternative gelling agent, which is increasingly used because it forms clear gels and is free of contaminating compounds. C. Teisson (pers. comm., 1988) found that 'Gelrite' gave better results than agar. 'Gelrite' is usually added at 2 g l^{-1}, i.e. one quarter of the highest agar concentration used (F. Novak, pers. comm., 1988).

The pH of media is usually kept at 5.8, using NaOH or HCl (0.1 or 1N) to make adjustments after all medium components have been mixed, and just prior to autoclaving. The author has observed that the pH drops by 0.5-1.0 unit during autoclaving, which sometimes affects the gelling efficiency of agar. The pH of the medium also changes during culture. Vuylsteke and De Langhe (1985) reported that the acidity of the medium increased to pH 4.5 during 1 month of culture, indicating the need for timely transplantation to fresh medium (see 4.1: Culture initiation).

3.3.2 *Media preparation*

Media are normally prepared by dissolving the appropriate chemicals in distilled water, adjusting the pH of the solution, adding agar (for semi-solid media) and autoclaving. MS medium can also be obtained from specialist suppliers as packets of premixed, powdered ingredients. A convenient procedure is to prepare media from concentrated stock solutions (Fig. 3). Table 3 gives the composition of the stock solutions and the amount of each required to prepare the different media involved in the micropropagation process (see 4: *In vitro* propagation methods).

Stock solutions of mineral salts are kept in dark bottles and stored in a refrigerator for up to 6 months. Stock solutions of vitamins and growth regulators are contained in 50 or 100 ml volumetric flasks. These are stored in the refrigerator for periods not exceeding 1 month. The growth regulator stock solutions have a concentration of 1 mM. However, for the preparation of multiplication medium, it is advisable also to keep a 10 mM BAP stock solution of which 2 ml are added to the medium.

A stepwise procedure for preparing 1 litre of medium is given in Fig. 4. All ingredients are added to distilled water and the solution is heated to dissolve the agar. Care should be taken not to add agar to boiling liquid as the medium is then liable to froth up and boil over. Once the agar has dissolved, the medium is distributed (ideally using a dispenser with adjustable volume) into culture vessels which are then capped. The amount of medium contained per culture vessel varies from 20 to 50 ml (see 3.4.1: Culture vessels).

Plant tissue culture media are usually sterilized by autoclaving at 121°C and a pressure of 1.05 kg/cm^2 (103.4 KPa) for 15-20 min. This period is adequate when

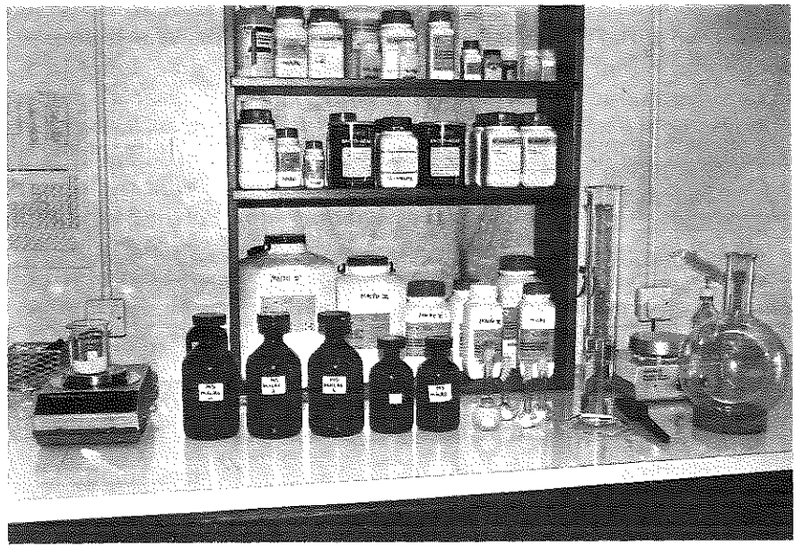


Figure 3. Medium preparation from stock solutions: From left to right: balance with sugar being weighed, MS mineral salt stock solutions in dark bottles, vitamin and growth regulator stock solutions in volumetric flasks, graduated cylinder with distilled water, pH stick, boiling flask.

media are dispensed into small lots, but needs to be increased with larger volumes of medium (e.g. when autoclaving is done before dispensing; Biondi and Thorpe, 1981). Most components in the medium are thermostable, but longer autoclaving times should be avoided wherever possible as they may induce chemical changes (Bonga, 1982). Thiamine and IAA are partly destroyed during autoclaving and can be sterilized by ultrafiltration (George and Sherrington, 1984). When this carried out, the medium must be sterilized in bulk, the additions made, and the medium then dispensed aseptically into pre-sterilized containers. If the medium is not to be used within 2 days, storage in a refrigerator is preferable (for up to 2 weeks). Care should be taken to label and date the containers of medium.

3.4 Culture environment

3.4.1 *Culture vessels*

Many different kinds of container can be used for banana and plantain shoot-tip cultures. There is ample choice between single-use and reusable culture vessels. Glass containers should, ideally, be made of borosilicate glass (e.g. 'Pyrex'). Such containers are costly but can be autoclaved and reused numerous times. Disposable and pre-sterilized plastic containers are becoming more widespread and greatly reduce the amount of routine washing. However, they can be prohibitively expensive.

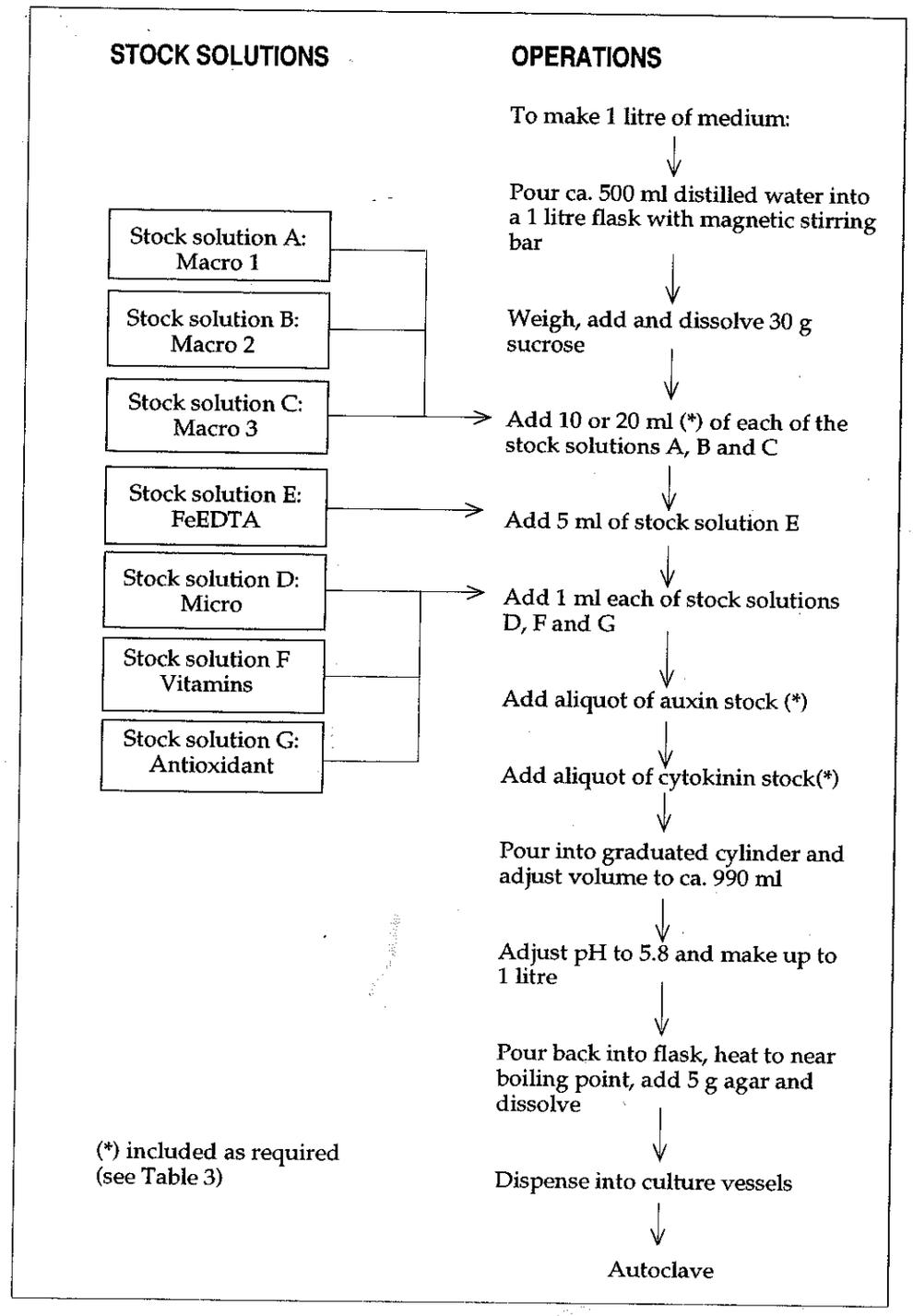


Figure 4. Schematic procedure for media preparation (as adopted for routine practice at IITA).

The volume of the container can sometimes affect growth *in vitro*. C. Teisson (pers. comm., 1988) observed that increasing the container volume enhanced growth and proliferation in *Musa* shoot-tip cultures. Good practice is to use progressively larger vessels at the different stages of micropropagation. The author uses test tubes (15 x 2.5 cm), containing 20 ml of multiplication medium, for culture initiation and multiplication. The same test tubes can be used for regeneration medium (20-30 ml). Alternative containers are Petri dishes (10 cm diameter), or wide-necked glass jars (11 x 6 cm). Rooted shoots are usually hardened in jars (50 ml of medium) or Petri dishes (30 ml of medium).

Test tubes can only hold one explant, while Petri dishes and jars may contain up to 10 explants each (see 4.3: Regeneration of plants). An advantage of Petri dishes is that up to five can be stacked on top of each other, resulting in a higher density of cultures per unit area. A culture shelf of 1 m^2 can hold 500 cultures in test tubes, 800 in jars, and 1100 in Petri dishes.

Test tubes can be closed by a variety of stoppers (e.g. made from cellulose) or autoclavable plastic caps (e.g. 'Kaputs' from Bellco Glass Inc.). Petri dishes are sealed with 'Parafilm' and glass jars by screwcaps or a double layer of aluminium foil.

3.4.2 *Culture room*

Tissue cultures are usually incubated in a room with controlled temperature and light regimes (also see 3.1.3: Culture incubation area).

The temperature should not fall below a minimum of 20°C or exceed a maximum of 35°C; the optimum incubation temperature is in the range of 26-30°C. Diurnal fluctuation between day and night temperatures of 32°C and 26°C may be expected when temperature is controlled by an air-conditioning unit. However, this satisfies the culture requirements of *Musa*.

Most micropropagation work is carried out under artificial lighting provided by cool-white, fluorescent tubes. A photoperiod of between 12 and 16 h light is widely used and is adequate for proliferative growth and regeneration of banana plants. Multiplying shoot-tip cultures have been maintained under a 24 h light regime, but once shoots had rooted, they were transferred to a light/dark cycle of 14/10 h (Vuylsteke and De Langhe, 1985).

Generally, a light intensity range of 1500 to 3000 lux is used, but higher light intensities promote more rapid plant regeneration (C. Teisson, pers. comm., 1988). Higher levels of 3000 - 10 000 lux during the final *in vitro* stage (Stage III; see 4.3: Regeneration of plants) also improve the survival rate of plantlets upon transfer to soil (George and Sherrington, 1984; Murashige, 1974).

3.5 Nursery area

An acclimatization treatment is necessary for the establishment of *in vitro*-produced plants in the non-sterile conditions of the external environment where the humidity is likely to be lower and the light level higher than *in vitro*. To achieve this *in vivo* establishment, a nursery area with some facilities for humidity control and partial shading is required. Conventional greenhouses, screenhouses or frames covered by a transparent polyethylene sheet are adequate for this. A temporary nursery can be constructed with bamboo sticks and palm leaves. Newly transplanted

banana plantlets are often kept in a mist bed under a low plastic cover before transfer to normal greenhouse conditions. Facilities for watering by a hosepipe or sprinkler must be available. Initially, shading of 50% is recommended and can be achieved by special shade cloth or palm leaves. Gradual hardening is then accomplished by progressively reducing the shade to expose the plantlets to full sunlight just before transplantating into the field.

Various types of nursery container exist; flats, plastic pots, fibre pots and polyethylene bags ('polybags') of different sizes are suitable. Black polybags are widely used at IITA because they are cheap and easy to handle during subsequent field planting. Plants grow best in large, 20 litre polybags (30 cm diameter, 30 cm high). Jarret *et al.* (1985) successfully used such bags. However, they occupy a lot of space in the nursery. Smaller 3.5 litre polybags (15 cm in diameter, 20 cm high) are preferred, because they take up only one quarter of the space, are much lighter to carry, and still allow vigorous plant growth. Handmade baskets woven from locally available palm leaf material are also very suitable and the plants can be field-planted directly, without removing the container.

Micropropagated bananas and plantains can be transplanted directly into soil (topsoil). An improved nursery medium would consist of a composted mixture of soil, sand and organic matter (O. Reuveni, pers. comm., 1987). The type of organic matter would depend on what is available locally (e.g. palm fruit pulp after extraction of the oil, chicken manure, coffee or rice husks). Suitable proportions of the components of the mixture need to be determined experimentally. F. Novak (pers. comm., 1988) routinely pots his plants in a 1:1 peat:soil mixture. Potting mixtures containing vermiculite have also been used (Gupta, 1986; Hwang *et al.*, 1984; Krikorian and Cronauer, 1984b) and can be purchased from horticultural suppliers. A supply of fertilizer helps to maintain rapid growth (see 4.3.3: *In vivo* establishment of plants).

A sterile soil or soil mixture is not essential for successful plant establishment, but is recommended to maintain the freedom from disease that can be achieved through *in vitro* procedures. Soil may contain insects, nematodes and disease organisms as well as weed seeds that can be eliminated by heating or fumigation. Jarret *et al.* (1985) fumigated soil with methyl bromide. At IITA, soil is sometimes pasteurized by heating an elevated drum filled with moist soil over a woodfire for 30 min at 60°C.

4. *IN VITRO* PROPAGATION METHODS

The *in vitro* production of plants proceeds through a sequence of three major steps as defined by Murashige (1974). Stage I: the initiation of an aseptic culture; Stage II: the multiplication of propagules, and Stage III: the regeneration of plants for transfer to soil. These three stages can also be distinguished in the shoot-tip culture procedure used for the micropropagation of bananas and plantains. They are described below. At each stage, the developmental pattern of the shoot-tip is manipulated by the medium composition, particularly by the growth regulator/s present. Thus, every stage requires a particular medium. In brief, shoot-tips derived from apical or lateral buds are induced to proliferate *in vitro* to form a mass of shoots or buds. These can be subdivided and further multiplied by subculturing, or rooted to obtain plantlets (Fig. 5).

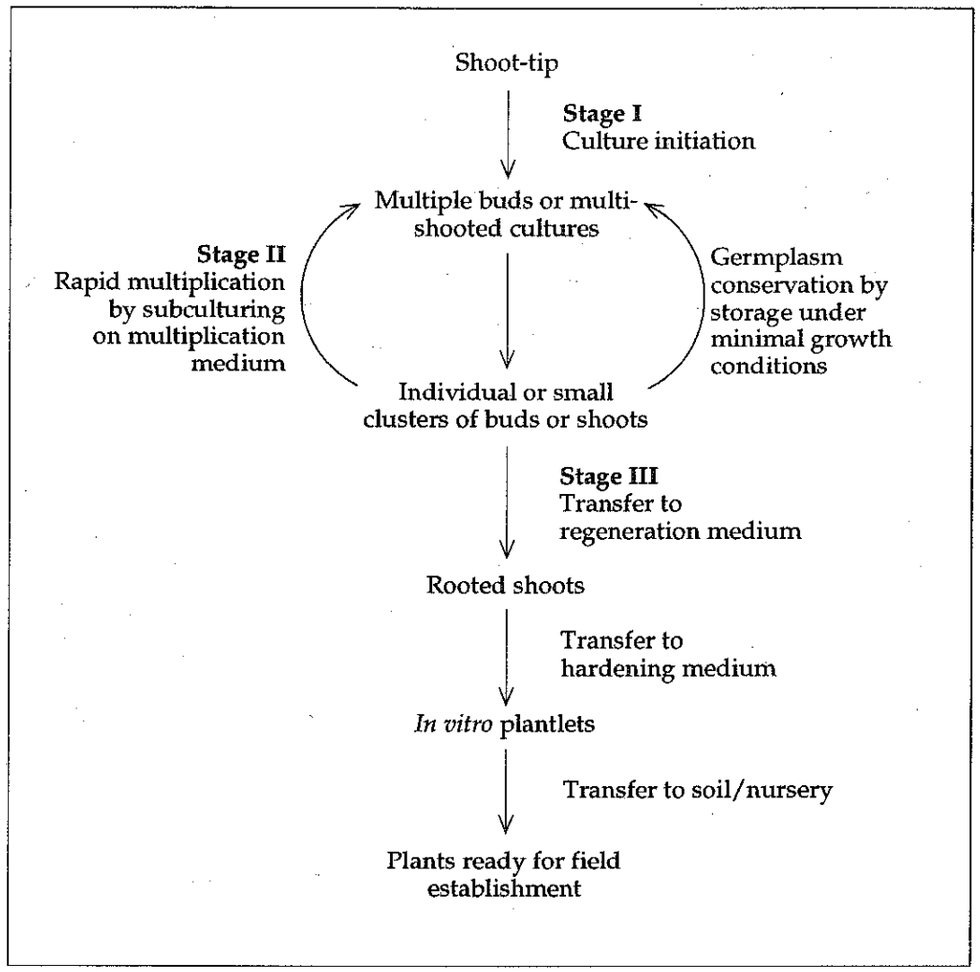


Figure 5. Schematic procedure for the production of banana/plantain plants by shoot-tip culture *in vitro*.

4.1 Culture initiation (Stage I)

Stage I in the micropropagation of *Musa* involves establishing an aseptic culture of shoot-tips. This is achieved by disinfection, excision and incubation of explants.

4.1.1 *Disinfection of explants*

Shoot-tips are preferably harvested from buds, peepers or small sword suckers (see 3.2: Plant material). Sword suckers are cut from source plants with a knife or a spade. This plant material is contaminated with micro-organisms, so it must be surface sterilized before the explants are transferred to culture. Shoot apices of bananas and plantains are enclosed in many tightly overlapping leaf initials. By virtue of this genuine natural protection against surface contaminants, the apices are easily sterilized. The sterilization procedure outlined below has been found to result

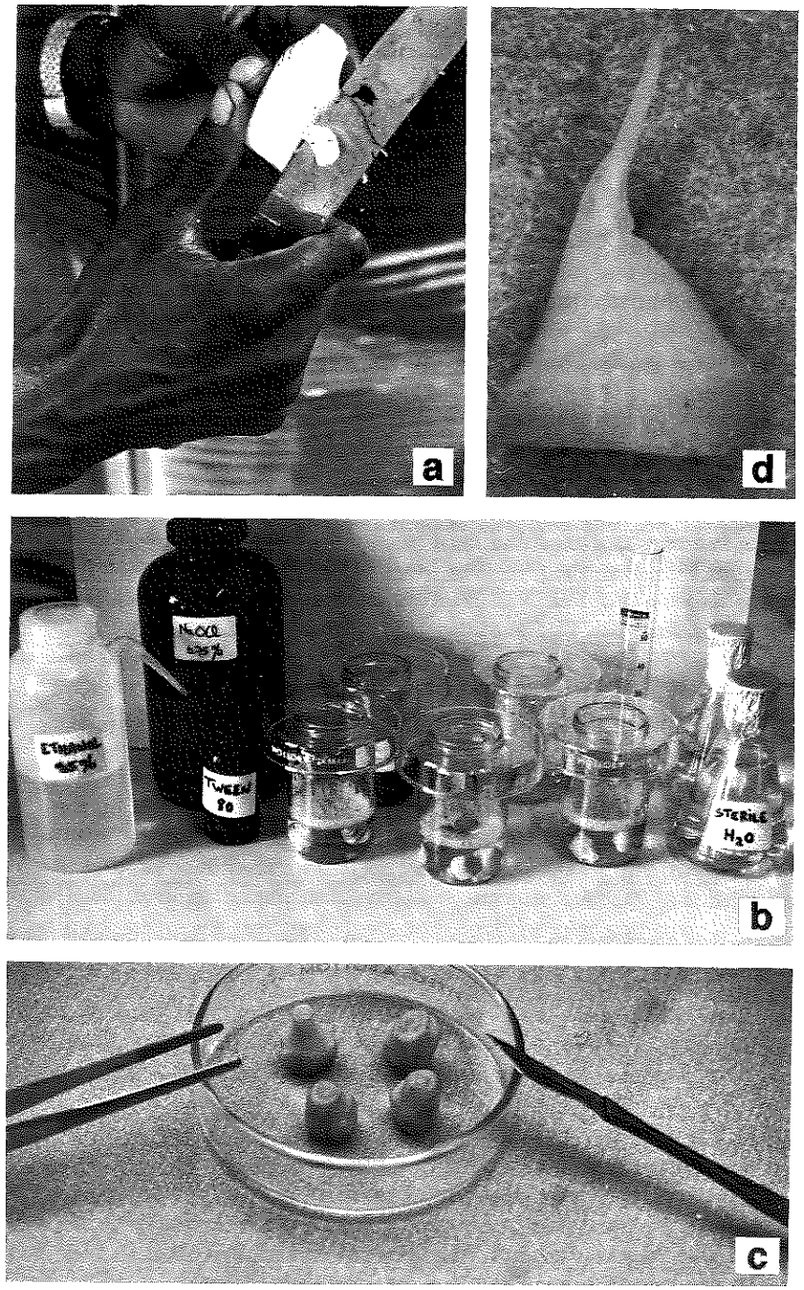


Figure 6 (Legend on opposite page)

in contamination rates of less than 5% in shoot-tips collected from field-grown plant material:

1. Remove superfluous tissue by trimming away the outer leaf sheaths, leaf bases and corm tissue until a 1-2 cm cube enclosing the shoot apex is obtained (Fig. 6a). Care should be taken to avoid cutting through the apex during this procedure.
2. Wash the tissue cubes under running tap water, rinse in 95% ethanol for 15-30 sec, and immerse for 15-20 min in a solution of bleach (0.75% (w/v) sodium hypochlorite (NaOCl); Fig. 6b). A wetting agent or liquid detergent ('Teepol', 'Tween 20' or 'Tween 80' at 1 drop per 50 ml) is added to the bleach solution to enhance penetration. Swirl the solution frequently.
3. Working under aseptic conditions, decant the bleach solution and rinse the cubes three times with sterile water (deionized or distilled and autoclaved). The cubes of tissue are now ready for excision of the shoot-tip (see 4.1.2 and Fig. 6c).

Variations on the above sterilization procedures have been proposed by several investigators. The concentration of NaOCl in the disinfecting solution is usually between 0.5 and 1.0%. If a laboratory grade NaOCl solution (usually 1N or 7.5%) is not available, a commercial laundry bleach solution is equally effective at a concentration of 10% (v/v), which gives approximately 0.5% NaOCl (Cronauer and Krikorian, 1984a; 1984b; Jarret *et al.*, 1985; Sandoval, 1985). (Note: A 1.5% (w/v) calcium hypochlorite ($Ca(OCl)_2$ solution, as used by Vuylsteke (1983) and Banerjee and De Langhe (1985), is as effective as 0.75% NaOCl).

Upper and lower limits for concentrations of the disinfectant are presented by Berg and Bustamante (1974), who initiated cultures without any disinfection procedure, and by Damasco and Barba (1984, 1985) who used undiluted bleach (5.25% NaOCl) for 45 min. The latter is a unnecessarily potent treatment in view of the shot apex's special morphology. If the explant is disinfected after excision (Cronauer and Krikorian, 1984a; 1984b; Vessey and Rivera, 1981), a shorter treatment time (5 min) and a lower hypochlorite concentration (0.0525% NaOCl) can be used (Krikorian and Cronauer, 1984b). However, surface disinfection before excising the shoot-tip is preferable, as then tissues that have been bleached and damaged by the hypochlorite solution can be cut away from the explant.

Some investigators apply a double-disinfection procedure (Novak *et al.*, 1986; Sandoval, 1985; C. Teisson, pers. comm., 1988). Firstly, larger tissue blocks 5-6 cm in diameter are soaked for 10-40 min in a solution of NaOCl of relatively high concentration (2.5-5.2%). The shoot-tip is then dissected to its final size and disinfected for a second time in a NaOCl solution of lower concentration (0.05-0.5%) for 10 min. Such intensive treatment is, however, unnecessary.

Figure 6. Disinfection and excision of explants for culture initiation: (a) Trimming of source material to obtain a cubical block of tissue containing the apex; (b) disinfection of tissue blocks in a 0.75% sodium hypochlorite solution; (c) surface-sterilized blocks of tissue in a sterile Petri dish ready for excision of shoot-tips, using forceps and a scalpel; (d) photomicrograph of a freshly excised shoot-tip ready for inoculation on to medium (shoot-tip is 5 mm long).

4.1.2 Excision of explants

The next step is the excision of the shoot-tip, which requires a great deal of patience and skill. Critical aspects of the process are the maintenance of aseptic conditions and the excision of the apex without excessive damage in order to avoid severe blackening. To ensure sterile conditions, shoot-tips are usually excised in a separate transfer area (see 3.1.2). Persons handling explants must obviously observe clean procedures and should not lean over the plant material. The operator's hands can be made relatively aseptic by washing with an antiseptic soap followed by spraying with ethanol (70-95%(v/v)). Sterilized parts of instruments or sterile plant material should not come into contact with the hands. During manipulations, dissecting instruments are repeatedly soaked in 70-95% ethanol followed by flaming. Instruments must also be autoclaved regularly, as some bacteria resist brief flaming. The excision procedure practiced in the author's laboratory is as follows:

1. The dissecting instruments are placed on a test tube rack in such a way that the sterile tips do not touch any surface. A 95% ethanol dip and an alcohol lamp are lined up with the rack holding the instruments for ease of repeated flaming (see Fig. 1a). Two scalpel holders with disposable blades, and two pairs of forceps, a longer round-tipped one and a short fine-tipped one, are used.
2. A sterilized cube of tissue is placed in a sterile Petri dish (Fig. 6c shows a number of cubes ready for excision). Holding the block steady with the forceps, the superficial tissue that was exposed to bleach during disinfection is cut away from the all sides of the cube.
3. Using a dissecting microscope or magnifier, the ensheathing cones of leaf primordia are systematically removed by carefully cutting with a scalpel through the circular insertion of each primordium, thereby loosening it from the basal corm tissue. Holding the corm piece with the forceps, the loosened outer leaf initial is pushed away. The precursory appendage of the inner initial often breaks during this process, but this is not harmful.
4. When the shoot-tip with its typical conical morphology is reduced to about 3-5 mm high, as much corm tissue as possible is trimmed from the base. Superfluous corm tissue will cause excessive blackening. At this stage, the explant (Fig. 6d) consists of the shoot apical meristem covered by 2-4 leaf primordia and supported on a small base of corm tissue. The excised shoot-tip is promptly transferred to a culture container and and pushed a short distance into the medium to ensure good contact. Shoot-tips are normally placed upright, although explant orientation is not really important.

Excised shoot-tips are often placed directly onto a medium that will encourage shoot bud multiplication (see Table 3), so that Stages I and II are not really separate. The growth of the shoot-tips following culture initiation is, therefore, described in the next section (see 4.2: Multiplication of propagules). For example, Krikorian and Cronauer (1984b) and C. Teisson (pers. comm., 1988) used the same medium (with high cytokinin content) for both culture initiation and continuous multiplication. Alternatively, one may establish cultures on a separate initiation medium, which typically has a lower cytokinin concentration than the multiplication medium, to which the cultures are subsequently transferred (Jarret *et al.*, 1985; Novak *et al.*, 1986; Sandoval, 1985).

The shoot apex is sometimes wounded by a series of cuts or fragmented longitudinally just before inoculation onto the medium. This practice, although not always necessary, is described below (see 4.2: Multiplication of propagules).

Explants can be pretreated with an antioxidant to reduce blackening of the explant itself and of the medium. Shoot-tips are briefly immersed in a sterile solution of 50 mg l^{-1} cysteine immediately after excision (Jarret *et al.*, 1985; Sandoval, 1985). Many variations of this pretreatment exist. Mante and Tepper (1983) placed explants of *Musa textilis* in an ascorbic and citric acid solution (1.0 and 1.5% (w/v)) respectively before surface disinfection. Novak *et al.* (1986) added the reducing agents citric acid (50 mg l^{-1}) and ascorbic acid (40 mg l^{-1}) to the disinfecting solution itself, while Gupta (1986) added 50 mg l^{-1} ascorbic acid to the sterile water used for rinsing the shoot-tips after disinfection, although reducing agents are more frequently incorporated into the culture medium (see 3.3.2: Media preparation; and 7.1: Blackening of shoot-tips).

The size of the explant is an important factor in the successful shoot-tip culture of bananas and plantains. Very small explants consisting of nothing more than the apical meristematic dome (less than 1 mm in height) increase the likelihood of producing virus-free plants, although they have a high mortality rate and grow very slowly (Vuylsteke and De Langhe, 1985). Explants that are too large (about 10 mm in height) are also unsatisfactory because they show more blackening and contamination, and thus lower resultant survival rates, than smaller explants (Sandoval and Müller, 1987). The latter authors concluded from a study with four cultivars of banana and plantain that the most appropriate explant size for culture initiation was 5 mm in height. From the literature, it appears that the most widely used explant consists of the apical meristematic dome covered by 1-4 leaf initials and measuring 2-5 mm in both height and diameter at the leaf base (Gupta, 1986; Jarret, 1986a; 1986b; Krikorian and Cronauer, 1984b; Novak *et al.*, 1986; Sandoval, 1985; Vuylsteke and De Langhe, 1985). Such explants have been proved to be excellent starting material for the establishment of vigorous cultures that multiply profusely.

4.1.3 Incubation of cultures

Following excision and inoculation, the cultures are labelled and then transferred to the culture room. Incubation conditions are discussed in detail in an earlier section (see 3.4: Culture environment).

4.1.4 Culture maintenance

Freshly excised shoot-tips are creamy white in colour, but the cut surfaces will invariably turn brown or black shortly after isolation. This blackening is caused by the oxidation of phenolic compounds upon wounding of the tissues. These compounds are exuded into the medium, are trapped by the agar and accumulate, forming a blackened area around the explant. This may interfere with nutrient uptake, resulting in inhibition of growth. Although many investigators anticipate this problem and perform an antioxidant pretreatment or routinely include antioxidants in the medium, the effectiveness of these treatments has been questioned (Jarret, 1986a; 1986b). The preferred and definitely the best method to control blackening at culture initiation is to transfer the explants to fresh medium after a short period of

time. This is usually done for the first time at 2-3 weeks after inoculation, but may have to be repeated regularly during the first 2 months. According to the author's experience, blackening is particularly intense in newly initiated cultures, and will decrease with time in culture for many cultivars.

Regular transfer to fresh medium is also recommended at Stages II and III. As growth proceeds, there will be increasing gradients of nutrients, growth regulators and metabolic waste products in the medium. The pH of the medium is also affected by time (see 3.3: Culture media) and concentrations of medium components change due to the evaporation of water. Transfer to fresh medium can be done every 3-5 weeks, although it has been noticed that well-established shoot-tip cultures of *Musa* are very hardy and can remain on the same medium for up to 2 months without any deterioration.

Contamination of cultures is unavoidable but generally occurs at tolerable rates (0-10%) if high standards of asepsis are maintained. Fungal and bacterial contamination usually appear within 5-10 days in culture. Cultures observed to be contaminated should be discarded. The problem of contamination is also discussed below (see: 7.2: Contamination of cultures).

4.2 Multiplication of propagules (Stage II)

Stimulation of multiple shoot or bud formation is achieved by culturing explants on medium supplemented with relatively high levels of cytokinin. This reduces the dominance of the apical meristem with the result that adventitious and/or axillary buds arise directly from the explant. The most widely used and most effective cytokinin for this purpose is BAP (see 3.3: Culture media), which is usually added at concentrations of 2-5 mg l^{-1}. BAP levels as high as 10 mg l^{-1} have also been used (Damasco and Barba, 1985; Dore Swamy *et al.*, 1983), but concentrations above 10 mg l^{-1} have been found to reduce shoot multiplication (Wong, 1986; Zamora *et al.*, 1986). BAP at 5 mg l^{-1} was found to be optimal by Cronauer and Krikorian (1984b), Jarret *et al.* (1985) and F. Novak (pers. comm., 1988), and can be considered to be the standard concentration for the induction of multiple shoots without associated root formation. The multiplication medium described in Table 3 has a BAP concentration of 20 µM (4.5 mg l^{-1}). Although adequate levels of shoot proliferation are achieved with cytokinin alone, auxin may be included (0.1-0.2 mg l^{-1}) as long as a high cytokinin to auxin ratio is maintained.

There is controversy as to whether the newly formed lateral meristems that arise directly from the explant originate adventitiously or from axillary buds. This is not surprising, if one considers that differing views already exist on the *in vivo* situation (Barker and Steward, 1962; Fisher, 1978) and that a truly complete anatomical investigation of the origin of *in vitro* proliferating buds has not been conducted. Nevertheless, Banerjee *et al.* (1986) demonstrated that proliferation in *Musa* ABB cv. 'Bluggoe' occurred through the formation of adventitious buds. 'Bluggoe' is one of the cultivars that shows very high rates of proliferation *in vitro* through the development of bulbil-like structures that bear numerous minute meristems.

Jarret (1986a; 1986b) argues that both budding systems, axillary and adventitious, occur in *Musa* shoot-tip cultures. Others have observed mixtures of both axillary and adventitious bud proliferation in one and the same culture

(F. Novak, C. Teisson, pers. comms., 1988). Under conditions of intensive multiplication on media containing high levels of cytokinin, the distinction between these two morphogenetic pathways may become unclear.

4.2.1 *Multiple shoot formation in excised shoot-tips*

A freshly excised explant can be inoculated onto the multiplication medium as an intact or fragmented shoot apex (see 4.1: Culture initiation). The latter technique of scoring the apex is commonly employed to reduce apical dominance, thereby stimulating the production of multiple shoot cultures. There are three practical approaches to apex wounding:

- Ma and Shii (1972) and Hwang *et al.* (1984) decapitated shoot-tips. However, such a radical procedure is not widely used and Wong (1986) reported that the removal of the apical dome is not essential for multiple shoot initiation.
- A series of vertical cuts into the meristematic dome were applied by M. Bustamante (pers. comm., 1983), Gupta (1986), Jarret *et al.* (1985) and Vessey and Rivera (1981). The number of incisions ranged from 2 to 10 and were made in such a way as to keep the base of the explant intact. This technique may increase explant and medium blackening greatly (Jarret *et al.*, 1985), although blackening may be prevented by the addition of ascorbic acid to the medium (Gupta, 1986).
- De Guzman *et al.* (1980) first described the procedure of splitting the shoot-tip longitudinally by cutting vertically through the apex and culturing the resulting halves or quarters as individual explants. This fragmentation method seems to be preferred by most investigators (Damasco and Barba, 1985; Epp, 1987; Jarret *et al.*, 1985; Cronauer and Krikorian, 1984b; Sandoval, 1985; Zamora *et al.*, 1986; Novak *et al.*, 1986). Jarret *et al.* (1985) reported that this method of shoot-tip fragmentation was more effective than the method of apical wounding alone, because it involved less blackening.

Scoring the apex may be done at the time of shoot-tip excision, but is more frequently performed 3-4 weeks after inoculation.

While fragmentation of the apex is performed in many laboratories, it is not essential for multiple shoot formation. Dore Swamy *et al.* (1983) noted no difference in response between intact and quartered shoot-tips. Satisfactory bud proliferation from intact shoot-tips has also been reported by Vuylsteke and De Langhe (1985), Banerjee *et al.* (1986), Wong (1986), and Zamora *et al.* (1986).

Intact shoot-tips cultured on multiplication medium will turn green in 7-15 days. At 3-4 weeks, the shoot-tip swells at the base. Upon removal of the outermost overarching leaf, a number of tiny meristems can be seen in an annular arrangement around the insertion of the inner leaf initial. Sequential removal of the remaining leaf initials will reveal more meristems, but their number and size decrease towards the central apical meristem (Banerjee *et al.*, 1986). Such meristems have been observed lying adaxially to the insertion of the second or third leaf primordium. This demonstrates the cytokinin-mediated suppression of apical dominance, because *in vivo*, the first distinct meristematic bud occurs distally to the insertion of the sixth to tenth leaf margin (Fisher, 1978).

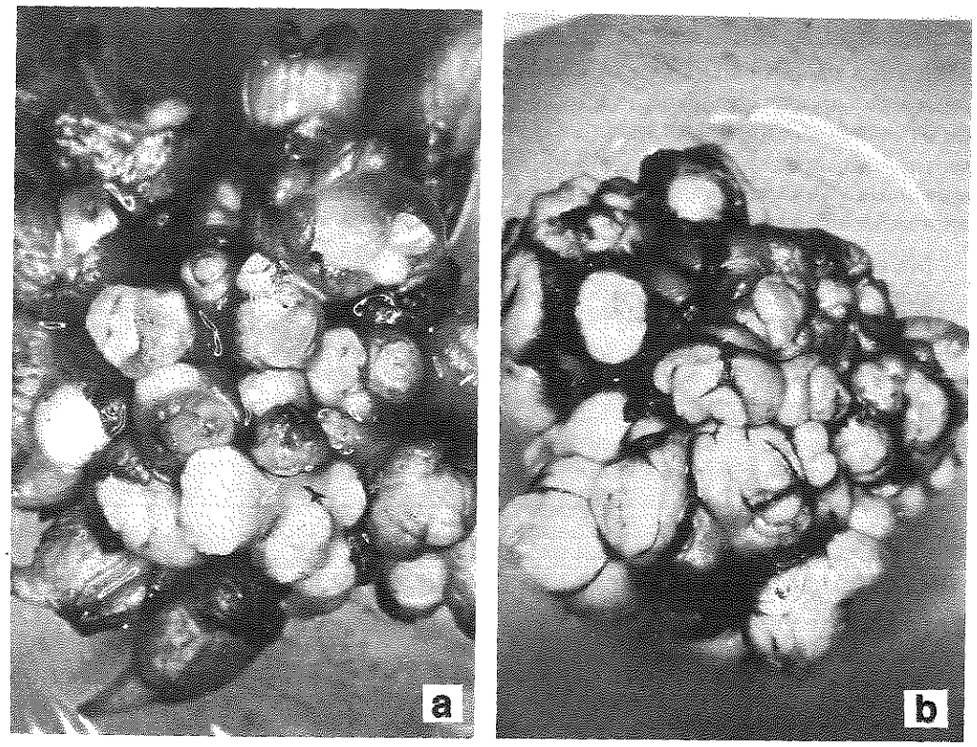


Figure 7. Highly proliferating shooot/bud cultures that appear as a clump of cormlets each bearing one or more meristems: (a) 1-year-old subculture of *Musa* (ABB) cv. 'Bluggoe'; (b) 2-year-old subculture of *Musa* (AAB plantain) cv. 'Agbagba' (diameter of tissue clumps is 2.5 cm).

Intact shoot-tips are generally left to grow for 6-8 weeks, during which time they may have been transferred to fresh medium several times (see above: Culture maintenance). The cultures will then appear as clusters of small shoots or as a clump of tiny corms (cormlets) that are covered by several minute meristem-tips. It may be necessary to remove the outer leaf to see these cormlets, although they often pierce through the ensheathing leaf. A morphological and histological study on the sequence of events leading to shoot bud proliferation during subculturing has been presented by Banerjee *et al.* (1986).

4.2.2 *Subculturing*

The multiplication of propagules is easily accomplished by subdividing the shoot or bud clusters with a few scalpel incisions and reculturing the smaller pieces (avoiding transfer of basal corm tissue) on fresh multiplication medium. The miniature

Table 4. Proliferation rates in shoot-tip cultures of some bananas and plantains, showing differences among cultivars.
Number of shoots/buds was generally counted following 6-8 weeks of culture on MS medium supplemented with 5 mg l^{-1} BAP.

Genome	Cultivar name	Shoot/bud increase	Reference
AAA	Lacatan	2.6	Wong (1986)
	Dwarf Cavendish	9.4	Banerjee and De Langhe (1985)
AAAA	IC 2	3.3	Wong (1986)
AAB	Mysore	6.6	Wong (1986)
	Silk	16.0	Banerjee and De Langhe (1985)
	Agbagba[1]	17.4	Vuylsteke (unpublished)
	French Sombre[1]	16.7	Vuylsteke (unpublished)
	N'Jock Kon[1]	19.5	Vuylsteke (unpublished)
	Asamiensa[1]	24.4	Banerjee and De Langhe (1985)
ABB	Cardaba	3.0	Zamora *et al.* (1986)
and BBB	Nzizi	5.7	Vuylsteke (see Fig. 8)
	Pelipita	18.0[2]	Cronauer and Krikorian (1984b)
	Bluggoe	20.4	Banerjee and De Langhe (1985)
	Saba	31.0	Jarret *et al.* (1985)

1. Plantains.
2. Split shoots after 4 weeks in culture.

propagules so obtained, which may consist of a single shoot/bud or a small group of them, will be induced to form new meristems. The result is a highly proliferative shoot/bud mass (Fig. 7a,b) and a very rapid rate of propagation. Through repeated subcultures, the multiplication of propagules proceeds in an exponential way until the desired number is achieved. An open-ended system can also be obtained by maintaining a continuing stock of proliferating cultures and transferring the newly produced shoot material to regeneration medium as needed. Aseptic conditions must be maintained during all of these culture manipulations.

Rates of multiplication of propagules range from 2 to 10 per month (Hwang *et al.*, 1984; F. Novak, C. Teisson, pers. comms., 1988). Table 4 gives the average multiplication rates of some banana and plantain cultivars. Rapidly growing cultures are normally subcultured at intervals of 4-5 weeks. If a culture appears as a mass of tiny cormlets, subculturing by subdivision is carried out about every 2 months, but such a long culture period requires a transfer to fresh medium midway through the interval.

Although subculturing can often be continued over many months and even years without adverse effects becoming apparent in culture, genetically variant shoots could arise through somaclonal variation (Scowcroft, 1984) and be multiplied undetected through several subcultures (see 7.4).

4.2.3 *Factors affecting multiplication rates*

Genotype:
Many investigators have encountered variation among cultivars in the degree and pattern of shoot bud proliferation *in vitro* (e.g. see Table 4). These differences have been observed not only among, but also within the different genomic groups of the Eumusa section of bananas. Presumably, variation in multiplication rate is due to different cultivar-dependent responses to cytokinin concentration in the medium. The varying degrees of *in vitro* bud proliferation suggest that levels of endogenous growth regulator differ between genotypes. (In this context, the terms genotype and cultivar are interchangeable since it is by virtue of genotype that cultivars differ (George and Sherrington, 1984).) Krikorian and Cronauer (1984b) suggested that differences in sucker production *in vivo* may be expressed faithfully, exaggerated, or even overcome under *in vitro* conditions. Vuylsteke and De Langhe (1985) could not determine a relationship between *in vitro* proliferation capacity and *in vivo* apical dominance. On the other hand, these authors proposed that the presence of a B genome in a cultivar's genomic makeup could play a role in the achievement of high proliferation rates. This is still not clear, because Zamora *et al.* (1986) observed large differences among cultivars within the same BBB (or ABB) group. Thus, it is suggested that the *in vitro* proliferation potential be tested before embarking on a large scale micropropagation operation with a new cultivar.

The pattern of shoot bud production may be related to the degree of proliferation. Highly proliferating cultures, such as those of the plantains (AAB) and many cooking bananas (ABB), appear as clumps of numerous bulbil-like structures or cormlets, each bearing one or several meristem-tips on their surface (Fig. 7b). Shoot outgrowth is largely inhibited in such cultures. Those with lower proliferation rates grow as clusters of miniature shoots in which leaf outgrowth is relatively rapid.

Cytokinin concentration and shoot-tip fragmentation
The single most critical medium factor in bud proliferation is clearly the cytokinin. Shoot-tip cultures of many banana and plantain cultivars proliferate profusely when cultured on multiplication medium containing 5 mg l^{-1} BAP. Cultivars exhibiting low proliferation rates through the formation of apically dominant shoots on this medium can be induced to proliferate more abundantly by increasing the BAP concentration above this standard level (Vuylsteke and De Langhe, 1985; Zamora *et al.*, 1986; also see Fig. 8). Evidently, the *in vitro* multiplication rate is a function of the cytokinin concentration. Each cultivar seems to have an optimum concentration for maximum response, beyond which the proliferation rate cannot be increased or will even decrease.

Another remedy for poor multiple shoot development is wounding of the explant (see 4.2.1: Multiple shoot formation in excised shoot-tips). As shown in Fig. 8, both apex wounding and increasing the level of cytokinin have approximately the same effect on cultures that do not proliferate well. Increasing the BAP concentration from 20 µM (control) to 50 µM significantly increased the proliferation rate in two-year-old subcultures of the ABB cooking banana cv. 'Nzizi'. A treatment involving two vertical apex incisions, followed by culturing on the standard medium with

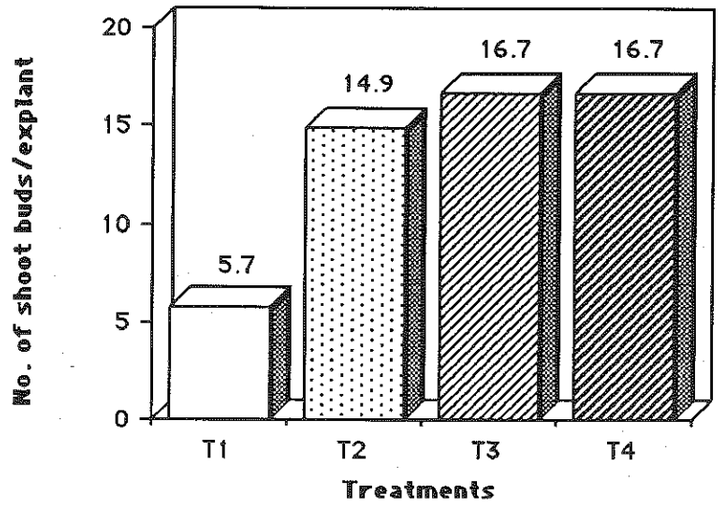


Figure 8. Effect of cytokinin (BAP) concentration and apex wounding on proliferation rates in shoot-tip culture of the *Musa* (ABB) cv. 'Nzizi'. T1: intact shoots on BAP 20 µM (control); T2: intact shoots on BAP 50 µM; T3: intact shoots on BAP 100 µM; T4: incised shoots on BAP 20 µM. Subculturing interval was 2 months; each treatment had 32 replicates. ($LSD_{0.05}$: 3.6).

20µM BAP, produced the same significant increase over the control. Further increasing the BAP concentration to 100 µM did not result in any significant improvement on the 50µM BAP treatment.

Explant size
Dore Swamy *et al.* (1983) and Epp (1987) reported that larger explants, consisting of the apical dome with 6-8 overlapping leaf bases, developed into multiple shoots more readily because they contained more lateral buds. However, initiating cultures from such large explants increases explant and medium blackening, thereby reducing the survival rate (Sandoval and Muller, 1987). The recommended size of the explant has been discussed earlier (see 4.1.2: Excision of explants).

For subculturing, C. Teisson (pers. comm., 1988) prefers to subdivide large multiple shoot clusters into smaller groups of 2-3 shoots, because these proliferate better than individual shoots. Another advantage is that such a subculturing procedure involves less manual labour.

Time in culture
There are indications that proliferation rates increase with the number of subcultures, i.e. with time in culture (Damasco and Barba, 1985; Jarret *et al.*, 1985; Zamora *et al.*, 1986). However, C. Teisson (pers. comm., 1988) reports that multiplication decreased in old cultures of cultivars with a B genome.

4.3 Regeneration of plants (Stage III)

In Stage III, steps are taken to grow individual plantlets and prepare them for adaptation to the external environment, because propagules multiplied during Stage II are very small and not yet capable of surviving in the soil. The main morphogenetic event to take place during this stage is the rooting of shoots. Where mainly buds were produced (see Fig. 7b), rooting will be preceded by the elongation of buds into shoots. Because shoot elongation and rooting can be accomplished on the same medium, they are not considered separately. Establishing rooted plantlets from isolated shoot-tips takes 7-10 weeks (Cronauer and Krikorian, 1984b; Vuylsteke and De Langhe, 1985).

4.3.1 *Shoot elongation and rooting*

In most cases, the presence of high cytokinin levels during Stage II inhibits root formation and a separate root-inducing medium has to be used at Stage III. Root initiation in micropropagated shoots, as well as elongation of buds into shoots, is easily accomplished by transferring propagules to the regeneration medium given in Table 3. As in subculturing, propagules may be individual shoots/buds or a small cluster of 2-5 shoots/buds. If larger culture vessels (e.g. Petri dishes, glass jars) are used, these can contain up to 10 shoots/buds each.

The cytokinin content of the regeneration medium, 1 µM BAP, is much lower than in the multiplication medium, so that the cytokinin/auxin ratio becomes ca. 1. Most investigators omit cytokinin entirely, although Gupta (1986) and Wong (1986) observed that it promoted rooting. Rooting has also been induced on basal medium devoid of any growth regulators (Cronauer and Krikorian, 1984b; Damasco and Barba, 1985; Jarret *et al.*, 1985; Sandoval, 1985; C. Teisson, pers. comm., 1988), but the author agrees with Jarret (1986a; 1986b) in recommending the inclusion of auxin.

The auxins most frequently incorporated into media to induce rooting are NAA, IAA and IBA. The author found that NAA (1 µM or ca. 0.2 mg l^{-1}) was more effective than IAA in increasing shoot elongation and subsequent rooting in plantain shoot-tip cultures. For example, in the plantain cv. 'Agbagba', more than 80% rooting was obtained in 2 months with NAA (Fig. 9), while IAA gave less than 50% success. Higher NAA concentrations (2 and 5 µM) did not give better results than 1 µM, but Mante and Tepper (1983) and Cronauer and Krikorian (1984b) found 1 mg l^{-1} (ca. 5µM) to be more satisfactory in *Musa textilis* and AAA and ABB bananas respectively. Because NAA (0.2-1.0 mg l^{-1}) readily induces rooting in many banana and plantain cultivars, it is the preferred auxin for the regeneration medium.

IBA was also noted as being effective at 1 µM (Banerjee and De Langhe, 1985; F. Novak, pers. comm., 1988; Vuylsteke and De Langhe, 1985) or even at 10-50 µM (Dore Swamy *et al.*, 1983; Mante and Tepper, 1983). Cronauer and Krikorian (1984b) reported no difference in the root-inducing effects of NAA, IAA or IBA in the presence of 0.025% (w/v) activated charcoal. Other investigators have also included 0.1-0.25% activated charcoal (Hwang *et al.*, 1984; Krikorian and Cronauer, 1984b; F. Novak, pers. comm., 1988; Sun, 1985). However, inclusion of activated charcoal may not be necessary; Cronauer and Krikorian (1984b) and Jarret *et al.* (1985) found that it failed to promote root initiation.

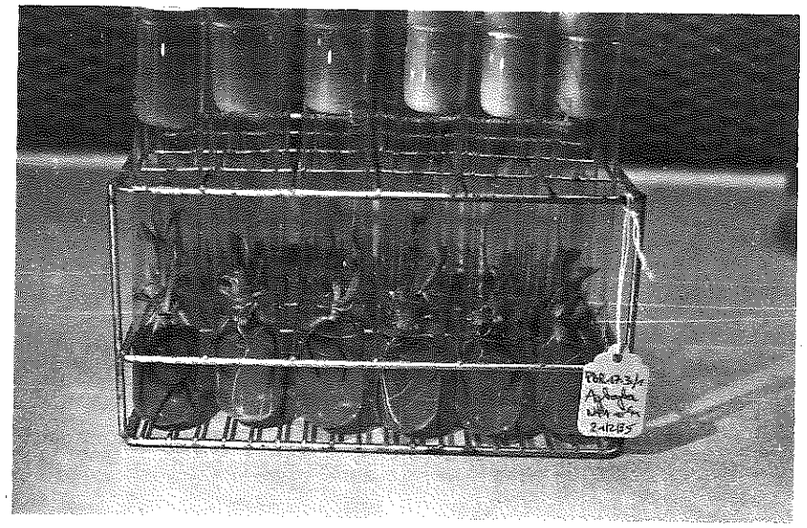


Figure 9. Regeneration of plants. Rooted shoots of plantain cv. 'Agbagba' after 6 weeks on regeneration medium.

Satisfactory rooting can occur on full strength MS medium, but in some laboratories it is common practice to induce rooting on medium with macronutrients at half concentration (Novak *et al.*, 1986; Vuylsteke and De Langhe, 1985). Mante and Tepper (1983) and F. Novak (pers. comm., 1983) also reduced the sucrose content to 1-1.5%.

Typically, white cord roots appear between 4 and 14 days after transfer to rooting medium if the Stage II propagules are leafy shoots at least 1 cm long. However, the roots can take up to 1 month to appear. It is clear that if the explants consist of bud-like structures (Fig. 7b), these will need to elongate first before rooting can be achieved. This explains the longer root-inducing periods (6-10 weeks) observed in banana and plantain cultivars having this pattern of proliferation (see 4.2.3: Factors affecting multiplication rates - genotype).

Once shoots have rooted, two options are available:

1. The rooted shoots can undergo an *in vitro* hardening period (see 4.3.2) prior to transplantation to soil.
2. The rooted shoots can be left for 2-3 weeks on the regeneration medium and then transferred immediately to the external environment (see 4.3.3: *In vivo* establishment of plants).

Which procedure to follow should be determined experimentally by comparing the survival rates upon transplantation to soil. Factors influencing these survival rates are the cultivar's behaviour *in vitro*, its ability to switch rapidly from largely heterotrophic to fully autotrophic nutrition, its resistance to abiotic stresses (low humidity, high light intensity) in the nursery, and the availability of sophisticated facilities for humidity and shade control in the nursery.

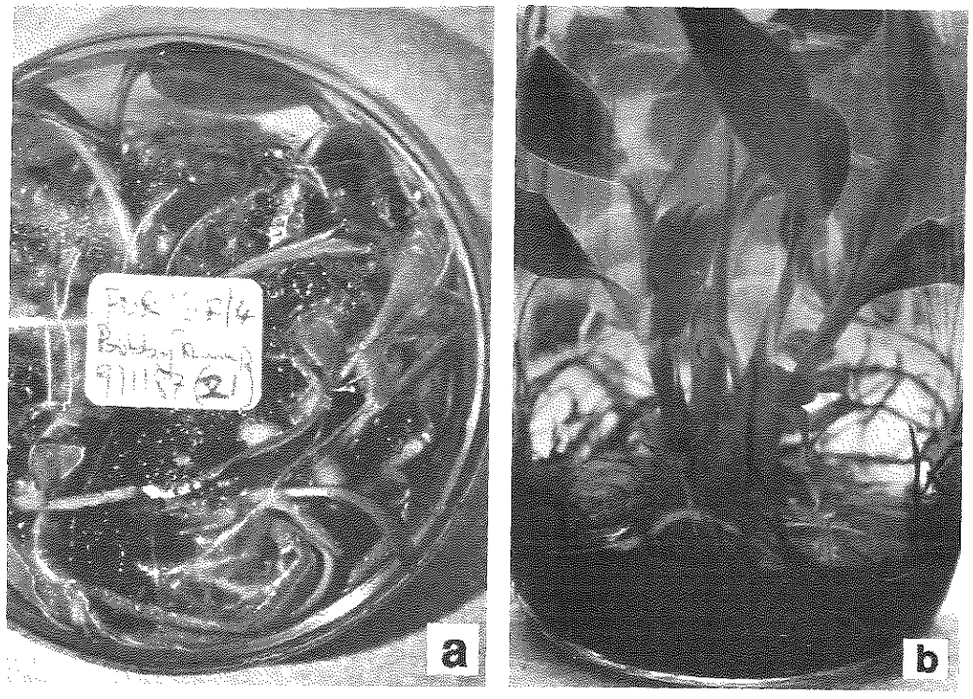


Figure 10. Regeneration of plants. (a) *in vitro* hardening of plantain plantlets, 4 weeks in Petri dish (Petri dish diameter is 10 cm.); (b) as a, in glass jar (jar is 11 cm high).

4.3.2 In vitro *hardening of plantlets*

In vitro hardening normally produces plantlets with well-proportioned shoots and roots that are capable of supporting each other and that have a survival rate in soil of over 90%. To this end, rooted shoots are transferred to hardening medium (Table 3), which is characterized by half strength macronutrients and the absence of growth regulators. Vigorous shoots 8-10 cm tall and supported by many long and profusely ramified roots typically develop within 3-5 weeks on this medium (Fig. 10a, b).

Many plantain cultivars generally exhibit slow shoot and root development once roots have been initiated. George and Sherrington (1984) pointed out that roots initiated by an auxin (particularly NAA) sometimes fail to grow in its presence, and shoot growth may be suppressed similarly. Even when shoots and roots continue to grow on regeneration medium, root ramification is rarely observed, resulting in reduced survival rates on transplantation to soil. However, survival rates exceed 95% if plantain plantlets are hardened *in vitro*. In contrast, shoots of many ABB-cooking bananas readily develop into sturdy plantlets with ramified roots when cultured on regeneration medium for 4-6 weeks. Such plantlets show a survival rate exceeding 90% when transplanted directly to soil.

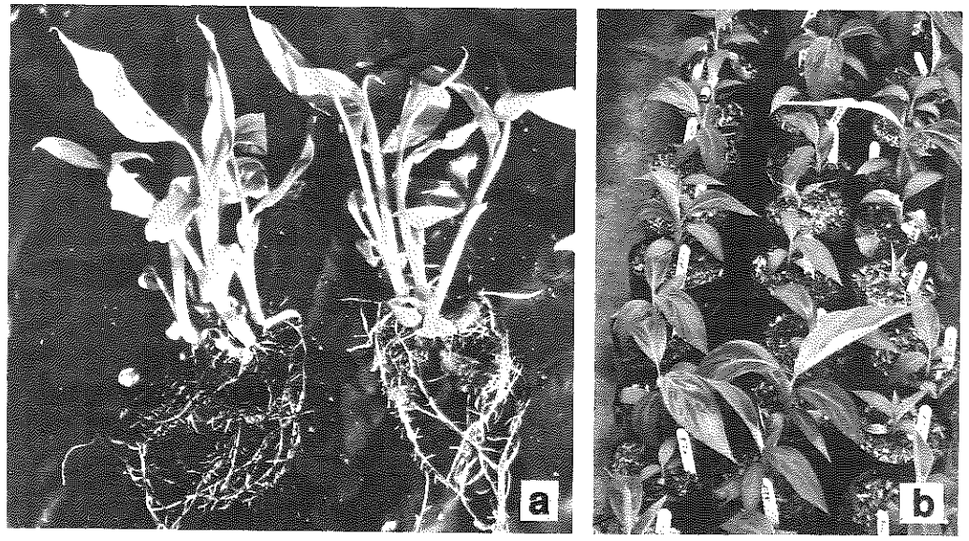


Figure 11. Establishment of *in vitro* plantlets in soil. (a) Clusters of plantlets ready for soil transplantation after separation into individual plantlets; (b) young plantain plantlets 1 month after transplanting (soil is contained in 3.5 litre polybags).

4.3.3 In vivo *establishment of plants*

The transfer of rooted plantlets from aseptic culture conditions to the external environment (Stage IV; George and Sherrington, 1984) can result in a significant loss of plants if not done carefully. Micropropagated plants are relatively delicate propagules because they have been grown in a high humidity and low light intensity. They may lose water rapidly upon transfer to natural conditions. Moreover, *in vitro* produced plants are believed to have a limited photoautotrophic capacity, so their energy demands must initially be met by reserves of starch accumulated during culture.

Rooted shoots that are 6-10 cm high and have several well-ramified roots are ready to be established in soil. As indicated earlier (see 3.5: Nursery area), topsoil or a composted soil mixture, preferably sterilized or pasteurized, are satisfactory planting media. Plantlets are removed from the culture containers and the agar is gently washed from the roots. If a cluster of plantlets is produced at the end of Stage III (Fig. 11a), this must be separated into individual plants by a careful scalpel cut through the basal corm tissue. Hwang *et al.* (1984) dipped banana plantlets in a fungicide solution prior to soil transplantation to reduce the risk of damage by fungal attack.

Individual plantlets are transplanted into the soil mix, taking care not to damage the fragile roots. The soil should cover the upper roots by 1-2 cm. After transplanting, plantlets are immediately watered. Although the soil mix must be kept moist initially, there is a narrow line between too much and too little. Jarret *et al.* (1985) argue that control of the soil moisture content is a critical factor in successful plantlet establishment. Maintenance of a high humidity is also important

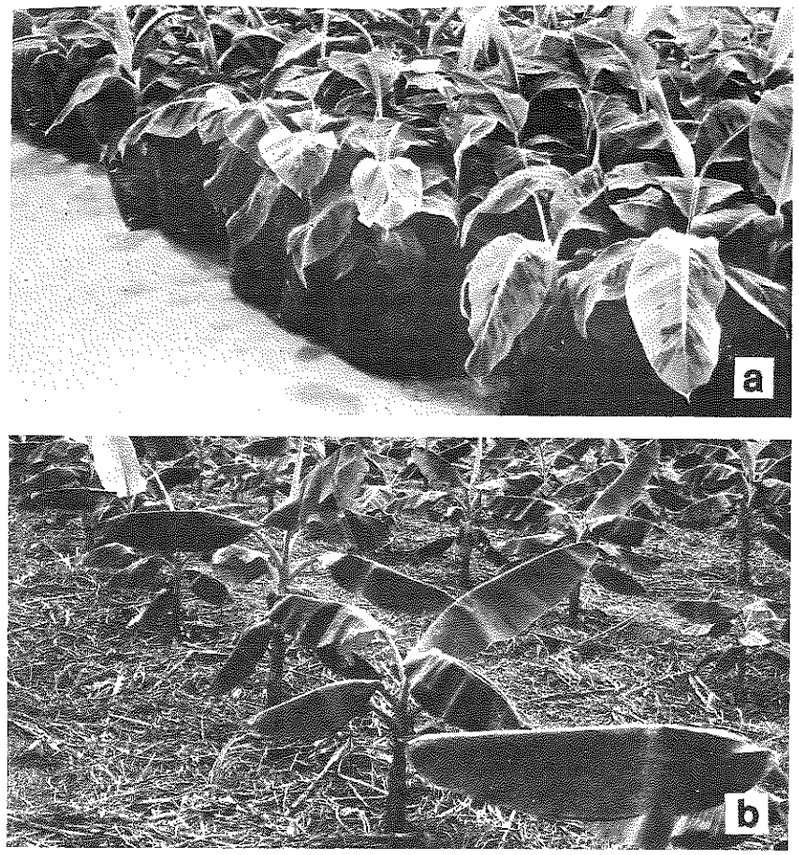


Figure 12. Establishment of *in vitro* plantlets in soil. (a) plantain plants, 2.5 months in the nursery (in 20 litre polybags), ready for field planting; (b) *in vitro* produced plantain plants, 3 months after field planting.

and can be achieved by intermittent misting or by keeping the plantlets under a relatively low plastic cover. Bower and Fraser (1982) covered plantlets individually with transparent polythene bags. Plantlets should also be kept in partial shade and hardened progressively by gradually reducing the humidity and shade. When these precautions are observed, survival rates should be 90-100%. It is beneficial to apply a dilute solution of fertilizer 4-6 weeks after transplanting (Fig. 11b). Urea (0.1-0.5 g per 100 ml per plant) and potash (KCl at 0.2-1 g per 100 ml per plant) are routinely applied by the author. Off-types that can be detected visually at this stage should be rogued.

Plants are generally allowed to acclimatize in the nursery for 2-3 months (Fig. 12a) and reach a height of 30-50 cm before transplanting into the field. Field preparation, planting and cultural management should be performed according to recommended practices (see Stover and Simmonds, 1987; Tezenas du Montcel, 1987) to achieve successful field establishment and subsequent vigorous growth (Fig. 12b).

5. *IN VITRO* CONSERVATION

Germplasm conservation of a vegetatively propagated crop like *Musa* in field collections is fraught with many problems (De Langhe, 1984). The use of shoot culture techniques to maintain such collections has considerable advantages. *In vitro* cultures occupy a relatively small amount of space (see 3.4: Culture environment), are suitable for international exchange if disease-free, and are amenable to rapid multiplication when necessary. A disadvantage is that some *in vitro* methods may lead to genetic instability (see 7.4: Somaclonal variation).

The simplest approach to *in vitro* germplasm storage would be to follow the procedure outlined above (see 4.2: Multiplication of propagules) in which multiple shoot cultures are maintained under normal conditions and subcultured every 2 months (Zamora *et al.*, 1986). However, this procedure entails frequent transfers, which are labour-intensive, time-consuming and increase the risk of loss due to contamination of cultures. Two approaches are envisaged for the storage of *in vitro* cultures that obviate the need for frequent manipulations: cryopreservation for long-term storage and slow growth for medium-term storage.

5.1 Cryopreservation

Little research has been done on the cryopreservation of *Musa* tissue cultures and the technique has not yet been applied successfully. Banerjee (1985) reported attempts to cryopreserve cooking bananas by the 'droplet freezing' method (Kartha *et al.*,1982) or by 'dry-freezing' (Withers, 1979). Survival levels of 80-90% were achieved when freezing was terminated after 15-30 min at -20°C. Cultures exposed to temperatures below -20°C invariably died. More research into cryopreservation is urgently needed (IBPGR, 1986).

5.2 Slow growth

Musa shoot cultures have been successfully stored under conditions of slow growth. Methods to reduce the growth rate *in vitro* involve modification of either the culture medium or the physical environment in which cultures are incubated. The latter seems to be the preferred approach. Banerjee and De Langhe (1985) maintained proliferating cultures of seven cultivars for more than 1 year at a low temperature (15°C) and low light level (reduced from 3000 to 1000 lux). These cultures were kept on a medium that contained 10 µM BAP. Jarret *et al.* (1986) stored 38 *Musa* accessions for up to 18 months at 18°C under a low light intensity. Their procedure is similar to that of Banerjee and De Langhe (1985), but cultures were maintained on filter paper bridges in liquid media. C. Teisson (pers. comm., 1988) has also stored cultures at 18°C for 9 months. Nuñez *et al.* (1986) observed that shoot cultures inoculated on MS medium with 0.5 mg l^{-1} BAP and kept at 14-16°C manifested almost no growth, but no mention is made of subculturing intervals.

An alternative but less common approach is to add osmotica to the culture medium. Zamora *et al.* (1986, 1987) stored cultures for up to 4 months on media containing sorbitol or mannitol (0.025 or 0.25 µM). Mora *et al.* (1986) achieved a 50% growth reduction by supplementing the medium with 4% mannitol and 3-9% sucrose.

Storage in slow growth for up to 18 months appears to be feasible (Jarret *et al.*, 1986). Cultures in storage should, nevertheless, be checked frequently for contamination or necrosis, and discarded or transferred to fresh medium as necessary.

6. GERMPLASM EXCHANGE USING *IN VITRO* CULTURES

Shoot cultures are very suitable materials for the local and international movement of *Musa* germplasm. In fact, they are now generally considered as the only acceptable material for the international transfer of banana and plantain germplasm (Frison and Putter, 1989; INIBAP, 1987b). *In vitro*-produced propagules are considerably lighter in weight and less bulky than conventional propagules, are amenable to rapid multiplication if required, and minimize the risk of inadvertent introduction of pests and pathogens. Due to the aseptic state of tissue cultures, many contaminants can be detected and discarded. However, the banana bunchy top virus (BBTV), causal agent of the Bunchy Top disease, appears to be able to pass undetected through *in vitro* culture, and is not necessarily eliminated by culture even in combination with thermotherapy. Rapid disease indexing methods for Bunchy Top are being developed, but are not yet available.

6.1 Technical guidelines

The FAO/IBPGR Technical Guidelines for the Safe Movement of *Musa* Germplasm (Frison and Putter, 1989) recommend the following procedures:

- It is recommended that all germplasm be transferred from one country to another as *in vitro* cultures.
- Meristem-tips should be cultured in the country of origin and sent to a transit centre. If this is not possible, vegetatively propagated material has to be sent to a transit centre where it will be subjected to meristem-tip culture. Each meristem of each accession has to be given a separate code number.
- Further multiplication will result in the production of replicates from each original meristem, some of which will be used for indexing, others for distribution.
- For material originating from America (where no BBTV is present), four *in vitro* plantlets are to be sent from the transit centre to an indexing centre where they will be indexed for CMV.
- For material originating from elsewhere, four *in vitro* plantlets are sent from the transit centre to two different indexing centres, of which one is to be a country not affected by BBTV, for indexing for BBTV and other viruses.
- For the movement of cultures, neither antibiotics nor charcoal should be added to the medium as they would hinder detection of bacterial contamination.
- As soon as the indexing centre(s) report negative results, the transit centre is then in a position to provide the *in vitro* duplicate of the particular meristem of the same code number to the region where the accession has been requested.
- All information on results of indexing of each accession should be recorded and stored in the transit centre.
- It is recommended that, once at its destination, the material be multiplied in field conditions under observation for a period of one year in agreement with the quarantine authorities of that region.

Presently, one transit centre, which also maintains a large *in vitro* collection, is operational: Laboratory of Tropical Crop Husbandry, Katholieke Universiteit Leuven (KUL), Kardinaal Mercierlaan 92, 3030 Heverlee, Belgium. More information can be obtained from this centre. Three indexing centres are recommended in the FAO/IBPGR guidelines: Quarantine station, Queensland Department of Primary Industries, Indooroopilly, QLD 4068, Australia; Department of Horticulture, Fruit Crops Division, UPLB, College, Laguna, Philippines 3720 and IRFA, CIRAD, BP 5035, Avenue du Val de Montferrand, 34032 Montpellier, France.

6.2 Practical guidelines

The following measures should be adopted to achieve the successful shipment of cultures:

1. The type of culture to be shipped should be chosen taking into consideration the facilities available to the recipient for handling *in vitro* material. If a tissue culture facility is available, it is preferable to send multiple shoot cultures (see 4.2: Multiplication of propagules). In the absence of such a facility, rooted shoots (see 4.3: Regeneration of plants) that do not require any further aseptic handling are selected.
2. The use of glass culture vessels should be avoided as they are liable to break. Plastic culture tubes with tightly fitting screw caps and plastic Petri dishes have given good results (F. Novak, C. Teisson, pers. comms., 1988).
3. Cultures to be shipped must be free from bacterial, fungal or viral infection. For details about virus indexing, refer to the FAO/IBPGR guidelines (Frison and Putter, 1989). Particular attention should be given to material originating in Southeast Asia, Australia or East Africa, where BBTV is prevalent.
4. For international shipment, arrangements have to be made with the quarantine officials who may issue a phytosanitary certificate. An import permit from the quarantine service of the receiving country is sometimes needed.
5. Whenever possible, cultures should be hand-carried. This is the method of choice and requires fewer precautions. Cultures can then be transported in the culture vessels in which they were grown. Vessels should be sealed with 'Parafilm' or tape. The containers should be kept upright in a box packed with shock absorbent material (e.g. paper towels, or polystyrene foam chips).
6. If the plantlets are to be mailed by air (surface mail is not recommended), special precautions must be taken. For the cultures to arrive undisturbed, they should be transferred to medium with an increased agar concentration (8-10 g l^{-1}) and contained in the smallest feasible culture vessel. Breaking up of the medium and its mixing with the cultures themselves are also less likely to occur if the free space between the culture and the vessel's closure is restricted. This can be done by filling up the vessel with more medium than usual or by inserting a sterile cotton plug to fill the space. Culture vessels should be tightly packed into a strong container containing shock-absorbent material as above. Roca *et al.* (1979) have ascribed partial losses of shipments by air to changes in the atmospheric pressure of the aircraft's mail compartment. Intercontinental shipments of *Musa* germplasm have also been lost due to contamination caused by fluctuating pressure in transit (J. Schoofs, pers. comm., 1988).
7. Each shipment should include a complete list of the shipped germplasm (species or cultivar name, genome and other pertinent information), a phytosanitary certificate and import permit if applicable, and instructions for subsequent handling by the recipient if necessary.

Several investigators have reported successful international movement of *Musa* germplasm in the form of shoot cultures (IITA, 1986, 1988; Jarret *et al.*, 1985; F. Novak, C. Teisson, pers. comms, 1988; Zamora *et al.*, 1986). Plants have been recovered from cultures that were in transit for 1-3 weeks. Up to 25 clones have been sent in a single shipment.

7. POTENTIAL PROBLEMS

Four problems can be encountered during the course of *in vitro* culture procedures or when recovering plants from culture: blackening of explants, contamination of cultures, culture deterioration and somaclonal variation.

7.1 Blackening of shoot-tips

Explants and media commonly brown and blacken during culture initiation and, to a lesser extent, during subculturing (see 4.1.4: Culture maintenance). Blackening is caused by oxidation of phenolic compounds in wounded tissues, and will first appear on the cut surfaces of freshly excised shoot-tips and subdivided shoot clumps. Blackening can be particularly severe in cultures of *Musa textilis* (Mante and Tepper, 1983), *Musa balbisiana* (C. Teisson, pers. comm., 1988) and some true Horn plantains (F. Novak, pers. comm., 1988). The author's experience confirms this.

To avoid loss of cultures due to blackening, it is recommended that a combination of the three treatments mentioned earlier (see 4.1.4: Culture maintenance) be applied. This is labour intensive but necessary to keep valuable material alive in culture:

1. Pretreat explants with an antioxidant by immersion in a sterile solution of cysteine, ascorbic acid or citric acid (alone or in combination), prior to inoculation on to the medium.
2. Include antioxidants in the culture medium (see 3.3.2: Media preparation). The addition of activated charcoal has also been proposed to prevent blackening in *M. balbisiana* cultures (C. Teisson, pers. comm., 1988) and in 'Williams' banana (Bower and Fraser, 1982).
3. Frequently transfer cultivars to fresh medium. When blackening is severe, cultures should be transferred at weekly intervals. As the degree of blackening becomes less, the transfer interval can be increased to 3-4 weeks.

The author has occasionally observed increased blackening when cytokinin levels in the medium were raised to stimulate bud proliferation. Thus, it may be beneficial to culture varieties prone to blackening on medium with BAP levels below 5 mg l^{-1}.

7.2 Contamination of cultures

To survive and grow properly, *in vitro* cultures need to be free of contamination by fungi and bacteria. Whilst contamination is unlikely if culture manipulations are performed under optimal conditions (see 3.1.2: Transfer area), this may not always be possible, and contamination of large numbers of cultures may sometimes occur. If contamination appears in a series of vessels inoculated the same day, this is likely to be due to failure to sterilize the dissecting instruments properly. In the author's laboratory, dissecting instruments wrapped in aluminium foil are regularly autoclaved in an attempt to avoid the loss of entire batches of cultures.

Culture vessels with large working surfaces, such as Petri dishes and jars with a wide mouth, are more liable to become infected during improper culture manipulations. Furthermore, because these larger vessels often contain more than one explant, many propagules may be lost. Contaminated cultures are usually discarded, but valuable material (e.g. a unique germplasm introduction) suffering from bacterial contamination may be rescued by including antibiotics in the culture medium. The following procedure, aimed at regenerating plants for establishment in soil and subsequent reintroduction *in vitro*, has proven to be effective (W. Dillemans, pers. comm., 1987):

1. Prepare the necessary amount of regeneration medium (see Table 3).
2. Dispense the medium into containers, autoclave, and allow to cool to 45-50°C.
3. Prepare a concentrated solution containing the antibiotics 'Cefotaximum' and 'Carbenicillin' to give final concentrations of 150 mg l^{-1} and 1000 mg l^{-1} respectively (e.g. each container of 20 ml of medium should contain 3 mg 'Cefotaximum' and 20 mg 'Carbenicillin').
4. Add the antibiotic solution to the containers via filter sterilization and under aseptic conditions, shake well, and allow the medium to cool.
5. Transfer contaminated cultures to the antibiotic-containing medium.
6. Repeat the above sequence until plantlets ready for soil transfer are obtained.
7. Establish recovered plants in soil.

Plant tissues are sensitive to antibiotics and prolonged exposure can result in the development of resistance through genetic change and/or selection (George and Sherrington, 1984). Thus, antibiotics should be used with care and only employed where alternative means of eliminating contaminants are not practical. For example, contaminated but vigorous shoot cultures can be disinfected with bleach (see 4.1.1: Disinfection of explants), and shoot-tips excised from them recultured *in vitro*. However, bleach may damage young cultures in which the meristems are only loosely protected by leaf material. For these, antibiotic rescue will be necessary.

7.3 Culture deterioration

During subculturing, most of the basal corm tissue attached to the subdivided shoot buds should be cut away. Not only will this reduce blackening, it will also reduce the chances of non-meristematic tissue proliferating and outgrowing the meristematic tissue.

7.4 Somaclonal variation

One of the most crucial aspects of *in vitro* culture for clonal propagation and germplasm conservation is the maintenance of genetic stability. However, somaclonal variation, which is genetic variation among plants regenerated from tissue cultures of a single parental clone, appears to be widespread. It is a potential hindrance to the *in vitro* propagation, conservation and exchange of germplasm (Scowcroft, 1984).

Table 5. The occurrence of phenotypic variation among *in vitro* micropropagated plants of banana and plantain

Genome	Cultivar	Variation frequency	Variant characteristics	Reference
AAA	Giant Cavendish	2.4%	dwarfism, narrow and drooping leaves, abnormal pseudostem colour, abnormal bunch	Hwang, 1986; Hwang and Ko, 1987
	Grande Naine	5-19%	dwarfism, abnormal fingers	Pool and Irizarry, 1987
	Grande Naine	7.2%	dwarfism, abnormal leaves	Reuveni *et al.*, 1985
	Grande Naine	25%	dwarfism, abnormal fingers, leaf abnormalities	Stover, 1987
	Williams	9.3%	dwarfism, red pseudostem colour	Reuveni *et al.*, 1985
AAB	Maricongo	21%	'French' reversion	Ramcharan *et al.*, 1985
	Dwarf Horn	38%	'French' reversion	Ramcharan *et al.*, 1985
	Agbagba	6%	'French' reversion, drooping leaves, distorted leaves	Vuylsteke *et al.*, 1987
ABB	Saba	0%	-	Stover, 1987

During the last few years, several reports of phenotypic variation among micropropagated plants of *Musa* have been presented (Table 5) and these confirm that it is a common phenomenon. (The author uses both terms 'phenotypic variation' and 'somaclonal variation' to describe culture-induced variability which is, with reasonable assurance, genetic in origin. This is determined by monitoring if variant traits are (1) transmitted through at least two ratoons, and/or (2) consistent among sucker progeny following a cycle of conventional clonal multiplication *in vivo* (Vuylsteke et al., 1988). Reports of frequencies of off-types range from as low as 2.4% (Hwang, 1986) to 25-30% (Müller and Sandoval, 1987; Stover, 1987) or even as high as 38% (Ramcharan *et al.*, 1987). Although these are relatively high

frequencies for plants regenerated from shoot-tip cultures, the spectrum of variant phenotypes is not very wide. Moreover, the majority of off-types are agronomically inferior to the parental clone.

For obvious morphological traits, the bulk of the variation parallels naturally occurring variability and should thus not be considered as being generated specifically by *in vitro* culture. The frequency of this variability is, however, considerably amplified *in vitro*. Dwarfism is the single most common variant trait (50-75% of the off-types) among *in vitro*-produced banana plants of the *Musa* AAA cv. 'Grande Naine' (Hwang, 1986; Hwang and Ko, 1987; Reuveni *et al.*, 1985; Stover, 1987). This cultivar belongs to the Cavendish subgroup of AAA bananas, in which dwarf mutations also commonly occur in nature (Stover and Simmonds, 1987). In micropropagated False Horn plantains, variation in inflorescence type in the form of reversion to a typical 'French' plantain bunch type accounts for 40-100% of the total variability (Ramcharan *et al.*, 1985; Vuylsteke *et al.*, 1988). 'French reversion' has also been observed *in situ* (Tezenas du Montcel *et al.*, 1983), but its occurence is greatly enhanced *in vitro*.

There is increasing evidence that the nature and extent of somaclonal variation is genotype specific in *Musa*. Stover (1987) noticed a frequency of off-types of 25% among *in vitro* propagated 'Cavendish' bananas, but none among 'Saba' (*Musa* ABB or BBB). Within the plantain subgroup, 6% phenotypic variation was observed in the False Horn cv. 'Agbagba' (Vuylsteke *et al.*, 1988), but 70% inflorescence variants were found in the False Horn cv. 'Bise Egome 2' (unpublished results). Such large cultivar-dependent effects suggest that there are inherently stable and unstable clonal lines. The latter may be in a more dynamic state of genetic flux, which becomes particularly apparent as a result of exposure to *in vitro* culture conditions (Scowcroft, 1985).

The frequency of somaclonal variation does not seem to relate to the length of time in culture (Reuveni *et al.*, 1986; Vuylsteke *et al.*, 1988). Rather, Reuveni *et al.* (1986) concluded that the major source of variants could be traced back to a few initial explants that mutated early in culture.

Measures to minimize variation have been proposed. A simple method is to rogue off-types that can be detected visually in the nursery. However, most variants can only be recognized at later stages in the field (Hwang, 1986; Pool and Irizarry, 1987). O. Reuveni (pers. comm., 1987) suggests limiting the number of plants produced from each explant (e.g. a maximum of 1000 plants per initial shoot-tip) to minimize chances that a single variant be multiplied unconsciously resulting in the loss of the original genotype. By adopting this measure, off-type frequencies have been kept below 5%, which is acceptable. Müller and Sandoval (1986; 1987) propose culturing shoots on MS medium containing 5 mg l^{-1} abscisic acid (ABA). This would induce the elongation of internodes and allow the regeneration of plants from nodal cuttings which contain only axillary buds. Plants regenerated from axillary buds are considered to be less likely to exhibit somaclonal variation (Scowcroft, 1984). However, this method has not yet been evaluated.

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APPENDIX I

List of *Musa* species and cultivars successfully propagated *in vitro* at the International Institute of Tropical Agriculture (Ibadan, Nigeria)

Genome and type	Name		
AA (wild)	*Musa acuminata*		*M. tavoy*
	M. acuminata (type 2)		*M. siam*
	M. acuminata (type 3)		*M. pahang*
	M. acuminata ssp. *malaccensis* (hybrid)		*M. basjoo*
	M. acuminata ssp. *malaccensis* (holotype)		
	M. acuminata ssp. *banksii*		
	M. acuminata ssp. *burmannica* (IV-9)		
	M. acuminata ssp. *burmannica* (Sinwobogi)		
	M. acuminata ssp. *burmannicoides* (Calcutta 4)		
	M. acuminata ssp. *truncata*		
	M. acuminata ssp. *zebrina* (Maioa)		
AA (clones)	Pisang lilin	Pisang tongat	Pisang madu
	Figue sucrée	Tjau lagada	SH 3142
	Akondro Mainty	Gwan Hour	SN 2
	SF 247	SF 248	SF 265
	Wh-o-gu		
AB	Kamaramasenge	Kisubi	
BB	*Musa balbisiana* (I-63)	*Musa balbisiana* (10852)	
	Musa balbisiana (Honduras)	*Musa balbisiana* (Montpellier)	
AAA (dessert bananas)	Giant Cavendish	Gros Michel	Poyo
	Pisang Nangka	Lacatan	Valery
	Km 5	Muga	Mattui
AAA (cooking and beer bananas)	Igisahira gisanzwe	Igitsiri	Intokatoke
	Nyamwihogora	Mbwazirume	Nakitengwa
AAAA	IC 2		
AAB (dessert bananas)	Pisang kelat	Silk	Guineo
	Figue Pomme 1	Prata	Popoulou
AAB (French plantains)	Bungaoisan	Akpakpak	Mbi Egome 1
	N'Jock Kon	French Sombre	Amou
	Obino l'Ewai	Apem Pa	Bobby Tannap

Genome and type	Name		
AAB (French plantains continued)	Madre del Platanar	Ntanga 2	Nadzia
	Ntanga 3	Mulolou	Bind Imossendjo
	Nazika	Gabon 2	Nyiretia Apem
	Congo 2	Gabon 4	Obubit Ntanga 1
	Medouma	85-03	Bise Egome 2
	Ntie	Lifongo	Kelong Mekintu
	Rouge de Loum	Nyombe 2	Rose d'Ekona
	Moutouka 1	Baka	Kar Ngou
	Moutouka 2	Kwa	Moto French
	Mbeta 1	Elat	Ndingo Likno
	Moungeli	Nguma	French rouge
	Msisa	Mzuzu	Walungu 1
	Walungu 8	Walungu 9	Walungu 15
	Walungu 16		
AAB (French Horn plantains)	Osoaboaso	Ntanga 1	Mbang Okon
	Batard	Diby 2	Nditu Ukom
	Ngomba	3 Vert	Plantain No. 3
AAB (False Horn plantains)	Agbagba	Apantu	Nyiretia Apantu
	Kaamenko	Orishele	Eba Oboikpa
	Borodehene	Red	Itu Iba Ukom
	Borodewuio	Didiedi	Atali Kiogo
	Niangafelo	Essang	Plantain No. 17
	Gabon 1	Gabon 3	Big Ebanga
	Moufoubila	Mbindi	Mimi Abue
	Mbouroukou 3	Walungu 7	Moto Ebanga
	Drooping leaf variant		
	Distorted lamina variant		
AAB (Horn plantains)	Ihitisim	Osakro	Asamiensa
	Daluyao	Ubok Iba	Nothing but green
	1 Hand plenty	Lysoka	Nothing but red
	3 Hand plenty	Mbomo	Elar Icon
AAB (other)	Laknau	Muracho	*Musa* x *paradisiaca*
	Ngougou	Tshambunu	*M. par. seminifera*
ABB (cooking bananas)	Bluggoe	Nzizi	Simili Radjah
	Fougamou 1	Saba	Pisang awak
	Pelipita 1	Monthan	Champa Masik
	Pelipita 2	Sabra	Pisang abu Perak
	Maduranga	Espermo	Ice cream
	Foulah 4	Bom	Gia Hui
	Cacambou	Kinkala	Gipungusi
Other	*Musa textilis*		

APPENDIX II

A summary of entries on *Musa* from the IBPGR *In Vitro* Conservation Databases (Wheelans and Withers, 1984)

Each entry includes five fields of data: CROP, naming the genus and species under investigation; NAME and ADDR, containing the name and address of the contact; DATE, indicating the year of the survey to whch information was submitted (1980/81, 1983, 1985 or 1987; there are some entries covering more than one year); and APPL, listing the interests of the contact and intended applications of the *in vitro* work. Full details of database entries, covering propagation, characterization, disease indexing, germplasm collecting, storage and exchange, and problems experienced, can be requested via IBPGR Headquarters.

CROP *Musa acuminata*
NAME CELIN, V.A.
ADDR Department of Agricultural Botany, College of Agriculture, Kerala Agricultural University, Vellayani, Trivandrum 695522, INDIA.
DATE 1987.
APPL Genetic manipulation.

CROP *Musa acuminata*
NAME CONTRERAS, I.
ADDR Dpto. de Botanica, Facultad de Ciencias Forestales, U.L.A. Merida 5101, VENEZUELA.
DATE 1985.
APPL Rapid clonal propagation.

CROP *Musa acuminata*
NAME THE DIRECTOR
ADDR Fruit Research Institute, Private Bag X11208, Nelspruit 1200, REPUBLIC OF SOUTH AFRICA.
DATE 1980/81.
APPL Rapid clonal propagation, virus or other pathogen elimination.

CROP *Musa acuminata*
NAME JARRET, R.L.
ADDR Apartado 78, CATIE, Turrialba, COSTA RICA.
(Present address: USDA/ARS, South Atlantic Area Regional Plant Introduction Station, 1109 Experiment St., Griffin, GA 30223-1797, USA).
DATE 1983.
APPL Fundamental research.

CROP *Musa acuminata*
NAME MA, S.S.
ADDR Dept. of Horticulture, National Taiwan University, Taipei, 107 Taiwan, CHINA.
DATE 1980/81.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm storage, germplasm exchange.

CROP *Musa acuminata*
NAME NAYAR, N.K.
ADDR Department of Agricultural Botany, College of Agriculture, Kerala Agricultural University, Vellayani, Trivandrum 695522, S. INDIA.
DATE 1987.
APPL Genetic manipulation.

CROP *Musa balbisiana*
NAME CONTRERAS, I.
ADDR Dpto. de Botanica, Facultad de Ciencias Forestales, U.L.A. Merida 5101, VENEZUELA.
DATE 1985.
APPL Rapid clonal propagation.

CROP *Musa balbisiana*
NAME JARRET, R.L.
ADDR Apartado 78, CATIE, Turrialba, COSTA RICA.
(Present address: USDA/ARS, South Atlantic Area Regional Plant Introduction Station, 1109 Experiment St., Griffin, GA 30223-1797, USA).
DATE 1983.
APPL Fundamental research.

CROP *Musa cavendishii*
NAME CHEN, W.H.
ADDR Taiwan Sugar Research Institute, 54 Shen Chan Road, Tainan 700, Taiwan, CHINA.
DATE 1985.
APPL Rapid clonal propagation, virus or other pathogen elimination.

CROP *Musa cavendishii*
NAME MA, S.S.
ADDR Dept. of Horticulture, National Taiwan University, Taipei, Taiwan, CHINA.
DATE 1985.
APPL Rapid clonal propagation.

CROP *Musa cavendishii*
NAME REUVENI, O.
ADDR Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan, ISRAEL.
DATE 1987.
APPL Rapid clonal propagation, germplasm storage.

CROP *Musa chinensis* (*Musa acuminata* cv. chinensis)
NAME SONDAHL, M.R.
ADDR Departmento de Genetica, Instituto Agornomico, Caixa Postal 28, 13100 Campinas, São Paulo, BRAZIL.
(Present address: DNA Plant Technology Corp., 2611 Branch Pike, Cinnaminson, NJ 08077, USA).
DATE 1980/81.
APPL Rapid clonal propagation (plant regeneration from callus), virus or other pathogen elimination, genetic manipulation.

CROP *Musa sapientum*
NAME REUVENI, O.
ADDR Agricultural Research Organization, The Volcani Centre, PO Box 6, Bet Dagan, ISRAEL.
DATE 1980/81.
APPL Rapid clonal propagation.
DATE 1983.
APPL Rapid clonal propagation.

CROP *Musa* spp.
NAME BANERJEE, N.
ADDR Laboratorium voor Tropische Plantenteelt, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3030 Heverlee, BELGIUM.
(Present address: Dept. of Botany, University of Calcutta, 35 Ballygunge Circular Rd., Calcutta 700019, INDIA).
DATE 1985.
APPL Fundamental research, rapid clonal propagation, germplasm storage, and germplasm exchange.

CROP *Musa* spp.
NAME BARBA, R.C.
ADDR Institute of Plant Breeding, University of the Philippines at Los Baños, College, Laguna, PHILIPPINES.
DATE 1980/81.
APPL Rapid clonal propagation, germplasm exchange.

CROP *Musa* spp.
NAME BATISTA TEIXEIRA, J.
ADDR CENARGEN-EMBRAPA, Avenida W5 Norte, Parque Rural, PO Box 10.2372, Brasilia, BRAZIL.
DATE 1983.
APPL Fundamental research, rapid clonal propagation, virus or other pathogen elimination, germplasm exchange, genetic manipulation.

CROP *Musa* spp.
NAME BHAGWAT, B.
ADDR Dept. of Plant Science & Biochemistry, University of the West Indies, St. Augustine, TRINIDAD.
DATE 1985.
APPL Rapid clonal propagation, genetic manipulation.

CROP *Musa* spp.
NAME BOLORUNDURO, M.O.
ADDR National Horticultural Research Institute, Federal Ministry of Science and Technology, Idi-Ishin, Jericho Reservation Area, P.M. B. 5432, Ibadan, NIGERIA.
DATE 1985.
APPL Fundamental research, rapid clonal propagation.

CROP *Musa* spp.
NAME BOWER, J.P.
ADDR Citrus and Subtropical Fruit Research Institute (CSFRI), Private Bag X11208, Nelspruit 1200, REPUBLIC OF SOUTH AFRICA.
DATE 1983.
APPL Rapid clonal propagation, virus or other pathogen elimination.

CROP *Musa* spp.
NAME BRAZIL, O.G.
ADDR Departamento de Quimica, IBBMA/UNESP, Caixa Postal 505, 18610, Botucatu, SP, BRAZIL.
DATE 1987.
APPL Fundamental research, genetic manipulation.

CROP *Musa* spp.
NAME DE GUZMAN, E.V. (deceased)
ADDR Bio-Science Building, Dept. of Horticulture, University of the Philippines at Los Baños, College, Laguna 3720, PHILIPPINES.
DATE 1980/81.
APPL Rapid clonal propagation, genetic manipulation.

CROP *Musa* spp.
NAME DE LANGHE, E.A.L.
ADDR Laboratory of Tropical Crop Husbandry, Catholic University of Leuven, Kardinaal Mercierlaan 92, B-3030 Heverlee, BELGIUM.
(Also INIBAP, Avenue du Val de Montferand, BP5035, 34032 Montpellier Cedex, FRANCE).
DATE 1980/81.
APPL Rapid clonal propagation, germplasm storage, germplasm exchange.
DATE 1987
APPL Fundamental research, rapid clonal propagation, virus or other pathogen elimination, germplasm storage, germplasm exchange.

CROP *Musa* spp.
NAME THE DIRECTOR
ADDR Plant Breeding and Genetics Section, Joint FAO-IAEA Division, PO Box 100, A-1400 Vienna, AUSTRIA.
DATE 1987.
APPL Genetic manipulation.

CROP *Musa* spp.
NAME DREW, R.A.
ADDR Redlands Horticultural Research Station, P.O. Box 327, Cleveland, QLD 4163, AUSTRALIA.
DATE 1987.
APPL Rapid clonal propagation, germplasm storage, natural and induced mutation studies.

CROP *Musa* spp.
NAME DUNCAN, E.J.
ADDR Department of Plant Science and Biochemistry, University of the West Indies, St. Augustine, TRINIDAD.
DATE 1985.
APPL Rapid clonal propagation, genetic manipulation.

CROP *Musa* spp.
NAME DUMORTIER, F.
ADDR Katholiek Universiteit Leuven, Kardinaal Mercierlaan 92, B-3030 Heverlee, BELGIUM.
DATE 1987.
APPL Fundamental research, rapid clonal propagation, virus or other pathogen elimination, germplasm storage, germplasm exchange.

CROP *Musa* spp.
NAME FITCHET, M.
ADDR Citrus and Subtropical Fruit Research Institute, Private Bag X11208, Nelspruit 1200, REPUBLIC OF SOUTH AFRICA.
DATE 1987.
APPL Fundamental research, rapid clonal propagation.

CROP *Musa* spp.
NAME GONZALEZ, L.G.
ADDR Escuela de Ciencias Agrarias, Universidad Nacional, P.O. Box 86, Heredia, COSTA RICA.
(Present address: IBPGR Regional Office for Latin America, CIMMYT, PO Box 6-641, Mexico 06600, DF MEXICO).
DATE 1987.
APPL Rapid clonal propagation, germplasm storage, germplasm exchange, genetic manipulation, *in vitro* collection.

CROP *Musa* spp.
NAME JADRAQUE, A.Q.
ADDR Twin Rivers Research Center, Madaum, Tagum, Davao, PHILIPPINES.
DATE 1987.
APPL Rapid clonal propagation.

CROP *Musa* spp.
NAME JARRET, R.L.
ADDR University of Florida, Tropical Research and Education Centre, 18905 SW 280th St., Homestead, FL 33031, USA.
(Present address: USDA/ARS South Atlantic Area Regional Plant Introduction Station, 1109 Experiment St., Griffin, GA 30223-1797, USA).
DATE 1985.
APPL Fundamental research, rapid clonal progagation, germplasm storage, germplasm exchange, genetic manipulation.

CROP *Musa* spp.
NAME KAMAU, H.N.
ADDR Kenya Agricultural Research Institute, Box 57811, Nairobi, KENYA.
DATE 1985.
APPL Rapid clonal propagation, germplasm exchange.
DATE 1983.
APPL Rapid clonal propagation.

CROP *Musa* spp.
NAME KIWIJAN, B.
ADDR Biology Department, Faculty of Science, Khon Kaen University, Khon Kaen 40000, THAILAND.
DATE 1987.
APPL Rapid clonal propagation.

CROP *Musa* spp.
NAME KRIKORIAN, A.D.
ADDR Department of Biochemistry, State University of New York at Stony Brook, New York 11794, USA.
DATE 1980/81.
APPL Fundamental research, rapid clonal propagation.
DATE 1987.
APPL Fundamental research.

CROP *Musa* spp.
NAME LITZ, R.E.
ADDR University of Florida, Tropical Research and Education Center, 18905 SW 280 St., Homestead, FL 33031, USA.
DATE 1980/81.
APPL Rapid clonal propagation, germplasm storage, genetic manipulation.
DATE 1983.
APPL Fundamental research, rapid clonal propagation, germplasm storage, genetic manipulation.
DATE 1987.
APPL Genetic manipulation.

CROP *Musa* spp.
NAME LOPEZ J., L.E.
ADDR Programa de Genetica Vegetal, ICA, Tibaitatá, AA 151123, El Dorado, Bogotá, COLOMBIA.
DATE 1987.
APPL Fundamental research, (physiology, morphogenesis), rapid clonal propagation, virus or other pathogen elimination, germplasm storage, germplasm exchange.

CROP *Musa* spp.
NAME MA, S.S.
ADDR National Taiwan University, College of Agriculture, Department of Horticulture, Taipei, Taiwan 107, CHINA.
DATE 1983.
APPL Fundamental research, rapid clonal propagation.

CROP *Musa* spp.
NAME MANZUR MACIAS, D.
ADDR Facultad de Agronomía, Universidad de Caldas, A.A. 275, Manizales, COLOMBIA.
DATE 1987.
APPL Fundamental research (morphogenesis), rapid clonal propagation.

CROP *Musa* spp.
NAME MASCARENHAS, A.F.
ADDR Biochemical Sciences Division, National Chemical Laboratory, Poona-411008, INDIA.
DATE 1983.
APPL Rapid clonal propagation, virus or other pathogen elimination.
DATE 1985.
APPL Fundamental research, rapid clonal propagation, virus or other pathogen elimination.

CROP *Musa* spp.
NAME MBWANA, A.S.S.
ADDR Tanzania Agricultural Research Organisation, Research Institute Maruku, PO Box 127, Bukoba, TANZANIA.
DATE 1983.
APPL Germplasm storage.
DATE 1985.
APPL Germplasm storage, germplasm exchange.

CROP *Musa* spp.
NAME MÜLLER L.
ADDR CATIE, Turrialba, COSTA RICA.
DATE 1980/81.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm storage.
DATE 1985.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm storage, germplasm exchange, genetic manipulation, cell culture.

CROP *Musa* spp.
NAME NE NSAKU, N.
ADDR IRAZ, B.P. 91, Gitega, BURUNDI.
DATE 1987.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm storage, germplasm exchange.

CROP *Musa* spp.
NAME NOVAK, F.J.
ADDR Plant Breeding Unit, IAEA Laboratories, A-2444 Seibersdorf, AUSTRIA.
DATE 1987.
APPL Rapid clonal propagation, germplasm exchange, genetic manipulation.

CROP *Musa* spp.
NAME OROZCO C., F.J.
ADDR CENICAFE, Chinchina, Caldas, COLOMBIA.
DATE 1985.
APPL Rapid clonal propagation, germplasm exchange.
DATE 1987
APPL Fundamental research, rapid clonal propagation, genetic manipulation.

CROP *Musa* spp.
NAME PEREIRA DA PAZ, O.
ADDR EMBRAPA/CNPMF, Cruz das Almas, Bahia, BRAZIL.
DATE 1987.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm storage, germplasm exchange.

CROP *Musa* spp.
NAME PHILIP, V.J.
ADDR Dept. of Botany, Calicut University, 673 635, Kerala, INDIA.
DATE 1985.
APPL Rapid clonal propagation.

CROP *Musa* spp.
NAME POLIUS, F.
ADDR Research and Development Division, Windward Islands Banana Growers Association (WINBAN), PO Box 115, Castries, St. Lucia, WEST INDIES.
DATE 1983.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm storage, germplasm exchange.

CROP *Musa* spp.
NAME REY, V.
ADDR ICA-CRI Tulenapa, Apartado Aéreo 22, Chigorodo, Antioquia, COLOMBIA.
DATE 1987.
APPL Plant pathology, virus detection in *Musa* plantlets produced *in vitro*.

CROP *Musa* spp.
NAME RASCO, E.T.
ADDR Institute of Plant Breeding, University of the Philippines at Los Baños, College, Laguna, PHILIPPINES.
DATE 1985.
APPL Rapid clonal propagation, germplasm storage, germplasm exchange.

CROP *Musa* spp.
NAME ROSTEN, A.R.
ADDR Del Monte Corporation, Agricultural Research Center, 850 Thornton Street, PO Box 36, San Leandro, CA 94577, USA.
DATE 1985.
APPL Rapid clonal propagation, germplasm storage and genetic manipulation.

CROP *Musa* spp.
NAME ROWE, P.
ADDR Division of Tropical Research, United Fruit Company, La Lima, HONDURAS.
DATE 1980/81.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm exchange.
DATE 1983.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm exchange.
ADDR FHIA (Fundacion Hondureña de Investigación Agrícola), La Lima, HONDURAS.
DATE 1985.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm exchange.

CROP *Musa* spp.
NAME SAHAVACHARIN, O.
ADDR Department of Horticulture, Faculty of Agriculture, Kasetsart University, Bangkok 10900, THAILAND.
DATE 1987.
APPL Rapid clonal propagation, genetic manipulation.

CROP *Musa* spp.
NAME SEBASIGARI, K.
ADDR IRAZ, B.P. 91, Gitega, BURUNDI.
DATE 1987.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm storage, germplasm exchange.

CROP *Musa* spp.
NAME SMITH, M.K.
ADDR Redlands Horticultural Research Station, PO Box 327, Cleveland, QLD 4163, AUSTRALIA.
DATE 1987.
APPL Rapid clonal propagation, germplasm storage, natural and induced mutation studies.

CROP *Musa* spp.
NAME SMITH, M.K.
ADDR Maroochy Horticultural Research Station, PO Box 5083, Nambour, QLD 4560, AUSTRALIA.
DATE 1987.
APPL Rapid clonal propagation, germplasm storage, germplasm exchange, genetic manipulation, production of off-types (somaclonal variants) from micropropagated bananas.

CROP *Musa* spp.
NAME TEISSON, C.
ADDR Institut de Recherches sur les Fruits et Agrumes, IRFA/CIRAD, BP 5035, 34032 Montpellier Cedex, FRANCE.
DATE 1983.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm storage, germplasm exchange, genetic manipulation.
DATE 1985.
APPL Fundamental research, rapid clonal propagation, germplasm exchange, protoplast culture.
ADDR CIRAD, Avenue du Val de Montferrand, BP 5063, 34032 Montpellier Cedex, FRANCE.
DATE 1987.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm storage, germplasm exchange, genetic manipulation, haploid production.

CROP *Musa* spp.
NAME TEZENAS DU MONTCEL, H.
ADDR IRFA/Neufchâteau, 97130 Capesterre Belle Eau, GUADELOUPE. (Present address: CIRAD, Avenue du Val de Montferrand, BP 5063, 34032 Montpellier Cedex, FRANCE).
DATE 1987.
APPL Rapid clonal propagation, virus or other pathogen elimination, germplasm storage, germplasm exchange, genetic manipulation, haploid production.

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