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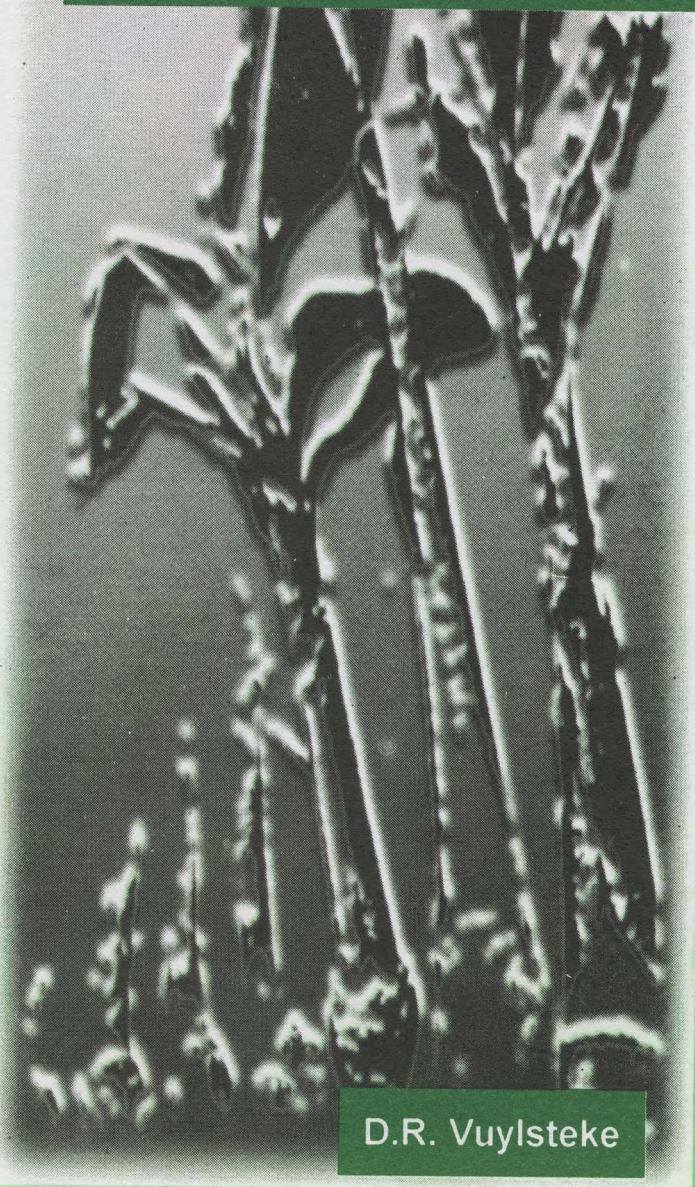
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Shoot-tip Culture
for the Propagation,
Conservation, and Distribution
of *Musa* Germplasm



D.R. Vuylsteke



**Shoot-tip Culture for the Propagation,
Conservation, and Distribution of
Musa Germplasm**

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Foreword

The first edition of this manual was published in 1989 by IBPGR (the International Board for Plant Genetic Resources; now IPGRI—the International Plant Genetic Resources Insti-

tute, Rome). Over the past decade, while the basic methods of micropropagation of *Musa* germplasm have not substantially changed, certain modifications and refinements have occurred. These have arisen partly as a pragmatic response to opportunities which new laboratory equipment and a wider range of materials and supplies offer for improving the efficiency of various protocols. In addition, new information was gained from continued experimentation in various aspects of *Musa* micropropagation and conservation leading to the modification of established protocols and the adoption of new ones. Also, during this period, handling of new germplasm emanating from *Musa* breeding programs has had some influence on established procedures.

Taking account of these several factors, the publication of this second edition is most timely. The author has made use of recently published papers on *Musa* micropropagation and incorporated recent revisions concerning safe movement of germplasm. Drawing on his own experience and his regular contact with other *Musa* scientists working in this field, he has achieved a comprehensive update of the first edition.

As applied when the first edition was published, micropropagation continues to play an important role in the conservation, multiplication, and distribution of *Musa* germplasm at national, regional, and international levels. It is hoped that this manual will assist practitioners to further improve and broaden their skills in this field.

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1. Introduction

Bananas and plantains (*Musa* spp. L.) are giant perennial herbs that thrive in the humid and subhumid tropics. They evolved from intra- and inter-specific hybrids of the two dip-

loid wild species *Musa acuminata* Colla. and *M. balbisiana* Colla. in the EuMusa series of the genus *Musa* (Simmonds and Shepherd 1955; Simmonds 1995). Its fruit provides one of the major commodities in international trade, but is far more important as a starchy staple in local food economies (Stover and Simmonds 1987; Robinson 1996). World production in 1996 was estimated at more than 85 million tonnes annually, of which about 10% entered the export trade (FAO 1997). Due to the economic and social importance of bananas and plantains, the conservation of *Musa* germplasm is a high priority (IBPGR 1981; Horry et al. 1997).

Musa production is threatened by pests and diseases, which have been increasing during the past 20 years. Most alarming has been the outbreak and spread of more virulent forms of the fungal diseases black sigatoka leaf spot (*Mycosphaerella fijiensis* Morelet) and fusarium wilt (*Fusarium oxysporum* Schlecht. f. sp. *cubense* [E.F. Smith] Snyd. & Hans.). Black sigatoka is now pantropic and can reduce yields by 30–50% (Stover 1983; Mobambo et al. 1993).

These problems have prompted an international response to increase efforts towards the genetic improvement of this important crop (Persley and De Langhe 1987; Rowe and Rosales 1996; Vuylsteke et al. 1997). As a result, there has been renewed interest in the collection, maintenance, and use of *Musa* germplasm. However, since most cultivated *Musa* are polyploid and vegetatively propagated, several difficulties arise in terms of germplasm handling and utilization:

- Germplasm exchange is impeded by the lengthy quarantine periods that conventional vegetative propagules must undergo due to their innate risk of disseminating pests and pathogens (Stover 1977; Chiarappa and Karpati 1984).

- Germplasm conservation in field genebanks is at risk of diseases, pests, and natural disasters, and requires large space and labor inputs (De Langhe 1984).
- Conventional propagation is slow, and suckers are bulky and generally of poor phytosanitary quality, which hampers the dissemination of selected (e.g., disease-resistant) cultivars or new hybrids.
- Genetic improvement by conventional hybridization is complex and difficult due to low seed fertility and the trisomic pattern of gene inheritance.

Yet, plant tissue culture techniques show great potential in enhancing the propagation, conservation, and genetic improvement of *Musa*, particularly as technical advances accumulated during the past 20 years (Krikorian and Cronauer 1984; Cronauer and Krikorian 1986; Stover and Buddenhagen 1986; Krikorian 1989; Vuylsteke 1989; Novak 1992; Israeli et al. 1995). Shoot-tip culture now is routinely and increasingly being used for the rapid clonal propagation, exchange, and conservation of a wide range of *Musa* genetic resources. The advantages of tissue culture micropropagation include higher rates of multiplication, production of clean or disease-free planting material, and the small amount of space required to multiply large numbers of plants. Although other in vitro culture systems also hold great promise, this manual only deals with shoot-tip culture. This is the only method currently used to produce tissue culture plants of many *Musa* genotypes in an easy, reliable, and routine manner.

2. Brief History of Shoot-tip Culture of *Musa*

Plant tissue culture is the term commonly used to describe the in vitro and aseptic cultivation of any plant part on a nutrient medium. Rapid clonal propagation was the first major practical application for 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 initial applications of tissue culture to *Musa*. The first reports of banana plants produced by shoot tip-culture came in the early 1970s from Taiwan (Ma and Shii 1972, 1974; Ma et al. 1978). These were shortly followed by Berg and Bustamante (1974) in Honduras, who used meristem culture combined with heat therapy to produce virus-free bananas. 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 early reports were based on research carried out on a small number of dessert banana cultivars of the Cavendish group (*Musa* AAA).

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 (Vessey and Rivera 1981; Vuylsteke 1983; Cronauer and Krikorian 1984a, 1984b; Banerjee and De Langhe 1985; Jarret et al. 1985; Vuylsteke and De Langhe 1985; Novak et al. 1986; Wong 1986; Vuylsteke 1989; Smith and Drew 1990a; Israeli et al. 1995). In vitro multiplication rates are several orders of magnitude higher than in conventional propagation. Plant production in vitro is mainly limited by the number of skilled technicians who can handle large numbers of shoot-tip cultures aseptically. Large-scale field establishment of micropropagated plants of banana and plantain, either for commercial or public research purposes, has been reported in Australia (Drew and Smith 1990), Cameroon (C. Teisson pers. comm. 1987), Colombia (R. Swennen pers. comm. 1987), Costa Rica (Jarret et al. 1985; Arias and Valverde 1987), Côte d'Ivoire (Kwa and Ganry 1990), Israel (Israeli et al. 1995), Jamaica (Oglesby and Griffis 1986; Stover 1987), Kenya (J. Robinson pers. comm. 1996), Morocco (Janick and Ait-Oubahou 1989), Nigeria (Vuylsteke et al. 1988, 1991, 1997), Philippines (Epp 1987; Zamora et al. 1989), Puerto Rico (Pool and Irizarry 1987; Liu et al. 1989), South Africa (Robinson et al. 1993), Taiwan (Hwang et al. 1984), Uganda, and US Virgin Islands

(Ramcharan et al. 1987). In the field, micropropagated banana and plantain planting material is capable of performance equal to or superior to conventional material (Smith and Drew 1990a; Vuylsteke 1998). In general, micropropagated plants establish more quickly, grow more vigorously and taller, have a shorter and more uniform production cycle, and produce higher yields than conventional propagules (Drew and Smith 1990; Robinson et al. 1993; Vuylsteke and Ortiz 1996). Yield gains from the use of in vitro plants can reach up to 20% in bananas and 70% in plantains, but such superior field performance is not always consistent and requires optimal crop husbandry (Vuylsteke 1998).

Aseptic shoot cultures also have been used as a vehicle for the international exchange of banana and plantain germplasm since the early 1980s (Jarret et al. 1985; Zamora et al. 1986). Since 1985, the International Institute of Tropical Agriculture (IITA) at Ibadan, Nigeria, has successfully used shoot-tip cultures, in combination with third-country quarantine, for interregional and intercontinental exchange of germplasm (Vuylsteke et al. 1990a,b). More than 300 *Musa* accessions were introduced from, and more than 100 distributed to, many parts of the world. These *Musa* genetic resources provided the basis for IITA's breeding program (Vuylsteke and Swennen 1993; Vuylsteke et al. 1997). The *Musa* Germplasm Transit Center of the International Network for the Improvement of Banana and Plantain (INIBAP) has exported more than 2500 accessions in shoot-tip culture form since 1985 (Van den houwe and Jones 1994). The INIBAP Transit Center is now the largest distribution source of *Musa* genetic resources, kept in tissue culture, in the world. Viruses are not effectively eliminated by tissue culture (Drew et al. 1989), hence, virus testing of germplasm now is recommended as a routine procedure to ensure its safe international distribution (Diekmann and Putter 1996). Reliable virus diagnostic techniques are available or being developed for the main known *Musa* viruses.

In vitro procedures have been used to facilitate the collection of *Musa* germplasm (Sharrock 1995), but there have been no reports of direct culture establishment in the field.

Tissue culture can also be applied to the conservation of *Musa* genetic resources. Shoot-tip cultures, often maintained at lower temperatures (15–18 °C) to reduce the need for subculturing, have been used for the slow-growth storage of *Musa* germplasm (Banerjee and De Langhe 1985; Jarret et al. 1986; Zamora et al. 1987; Van den houwe et al. 1995). The INIBAP Transit Center holds more than 1000 different *Musa* accessions using the method of slow-growth shoot-tip cultures (Van den houwe

and Jones 1994). This in vitro collection is the largest in the world and represents the greatest part of the existing genetic diversity in the genus *Musa*. Recently, a simple technique was developed for the cryopreservation of banana meristem cultures, involving preculture on high-sucrose media followed by rapid freezing (Panis et al. 1996). This method should greatly facilitate the long-term conservation of *Musa* genetic resources.

This short overview of developments during the past 25 years emphasizes the great value of in vitro culture techniques as enabling tools in the handling of *Musa* germplasm. As shoot-tip culture is a simple technique by which different types and cultivars of bananas and plantains have been micropropagated on defined culture media, it is recognized as a reliable and reproducible procedure for handling *Musa* germplasm in vitro.

3. Materials and Equipment

Successful shoot-tip culture results from the correct interaction between plant material, culture medium, and culture environment. These factors are discussed below. Procedures involved in the shoot-tip culture of *Musa* are covered in the following section (4: Micropropagation Methods).

3.1 Laboratory facilities

Ideally, a plant tissue culture facility consists 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 media. 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 (brown) storage bottles. Culture vessels may be of glass or disposable material (3.4.1 Culture containers). A hot and cold water supply, 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 deionized or distilled water is desirable, since tap water is generally considered unsuitable for plant tissue culture media. The most common and preferred method of water purification for tissue culture use is a deionization treatment followed by one or two glass distillations. Reverse osmosis purifying equipment combined with membrane filtration is used in some laboratories for the production of very pure water, but such sophistication is not really necessary for routine

micropropagation. Also, if a source of clean (tap) water is available, this could be used directly in the preparation of media for routine micropropagation, saving costs on purification apparatus (Ganapathi et al. 1995).

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 microorganisms 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. 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 1a. Set-up of the laminar air flow cabinet for aseptic manipulations. Arrangement without magnifying aid and electric, dry-heat, glass bead sterilizer to disinfect the metal dissecting tools.

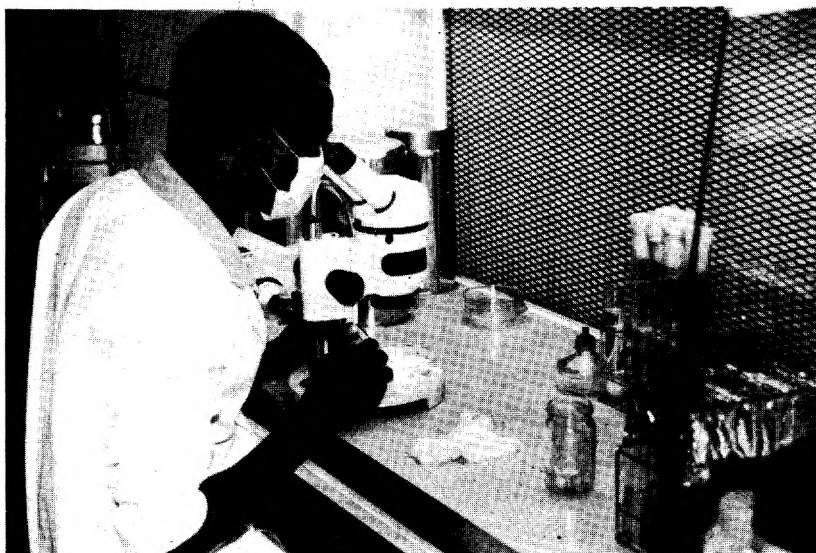


Figure 1b. Arrangement with stereomicroscope. From left to right: sterilizing boxes containing sterile petri dishes, light source, stereomicroscope, alcohol lamp, ethanol dip with dissecting instruments on a test tube rack, medium in test tubes and glass jars.

Small equipment and tools needed include an assortment of dissecting instruments (scalpels with removable blades, and forceps), a spray bottle of ethanol, and an ethanol dip with alcohol-lamp or bunsen burner in case the dissecting tools are sterilized by flaming. An electric, dry-heat, glass-bead sterilizer is used to disinfect the metal dissecting tools in the more sophisticated laboratory (Fig. 1a). Aseptic manipulations of *Musa* shoot-tip cultures generally do not require a magnifying aid. However, in some laboratories or for certain manipulations (e.g., meristem culture and embryo dissection from seed), a stereomicroscope with light source (Fig. 1b) or magnifier mounted on a flexible arm is used to work with very small pieces of tissue.

3.1.3 Culture incubation area

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

Cultures are normally kept in culture tube racks or trays on shelves illuminated by fluorescent lamps. Artificial light is usually provided by

40 W cool-white fluorescent tubes. Ballast devices in the fluorescent lamps are often installed outside the culture room as they generate heat. The photoperiod is preferably controlled by means of time switches installed in each culture room or on each set of shelves. For maximum use of space, a rack of shelves can be mounted on wheels so they can be placed side by side. At IITA, a culture room of 10 m² 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 of the culture room are given elsewhere (3.4 Culture environment).

Further reading on laboratory facilities is available in Biondi and Thorpe (1981), Brown and Thorpe (1984), George and Sherrington (1984), 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). The physiological and ontogenetic age of the shoot tip apparently does not influence the behavior 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 minimum damage being inflicted to the parent mat during their removal.

Terminal inflorescence apices (the tip of the male bud) and young flower buds in bract axils also are capable of producing plants using in vitro protocols similar to the shoot-tip culture technique (Ma et al. 1978; Bakry et al. 1985; Cronauer and Krikorian 1985a,b, 1988; Dore Swamy and Sahijram 1989). The terminal floral apex and the axillary flower buds manifest morphogenetic plasticity in their juvenile stage and can be induced to revert to vegetative growth, producing multiplying shoots in vitro.

Explant material should preferably be collected from flowering plants in order to ascertain trueness to type. Source plants also should be apparently healthy and grow vigorously. Special pretreatments of the mother plant are not required.

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



A wide range of species, landraces, and cultivars, representing the major part of the variability within the genus *Musa*, have been successfully propagated in vitro. At IITA, the author has micropropagated more than 400 different genotypes of wild and cultivated *Musa* of all genomic constitutions (AA, BB, AAA, AAB, ABB, AAAA, AAAB, AB BB, etc.; e.g., Vuylsteke et al. 1990a). 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 4–6 different genomic groups. Van den houwe and Jones (1994) report on the in vitro culture of 1056 accessions for the purposes of germplasm distribution and storage. New hybrid genotypes of various ploidies, produced by different breeding programs, have, of recent, also been propagated on a large scale successfully. All these reports indicate that current tissue culture procedures are applicable to all *Musa*. [Note: Ploidy and

genomic constitution vary among the different cultivars and groups of bananas and are designated by A and B to represent the genomes of *M. acuminata* and *M. balbisiana*, respectively. The putative genome of a particular cultivar or accession is derived from a diagnostic scoring method of phenotypic descriptors (Simmonds and Shepherd 1955; Stover and Simmonds 1987)].

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; Table 1) is very suitable for banana and plantain shoot-tip culture. It is the most widely used medium. Some authors (Ma and Shii 1972; Hwang et al. 1984; Epp 1987) report the use of a Smith and Murashige (1970) medium, in which the salt composition is identical to that of MS, except for the additional phosphate. Most investigators use slightly modified MS medium, with alterations only to the organic constituents (carbon, vitamin, amino acid, and growth regulator supplements; Table 2). Marchal et al. (1988) suggest the use of MS medium enriched with P and K to produce stronger in vitro plants. As a guide, one such modified, effective 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), with 3% (30 g l⁻¹) being most common. Sucrose of analytical grade is expensive and can be substituted by refined grocery sugar (e.g., Ganapathi et al. 1995). During culture, the sucrose hydrolyzes into fructose and glucose (Marchal et al. 1988, 1992). Replacement of sucrose by similar concentrations of fructose, alone or in combination with glucose, increases the fresh and dry weights of in vitro plants, but fructose is more costly than sucrose (Marchal et al. 1992). Sucrose can also be substituted by dextrose at the same concentration (De Guzman et al. 1980; Krikorian and Cronauer 1984b).

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 also be beneficial to the growth of banana

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

Major salts Salt	mg l ⁻¹	Minor salts Salt	mg l ⁻¹
KNO ₃	1900	MnSO ₄ ·4H ₂ O ^z	22.3
NH ₄ NO ₃	1650	H ₃ BO ₃	6.2
CaCl ₂ ·2H ₂ O	440	ZnSO ₄ ·4H ₂ O	8.6
MgSO ₄ ·7H ₂ O	370	KI	0.83
KH ₂ PO ₄	170	Na ₂ MoO ₄ ·2H ₂ O	0.25
FeSO ₄ ·7H ₂ O	27.8	CuSO ₄ ·5H ₂ O	0.025
Na ₂ EDTA·2H ₂ O	37.3	CoCl ₂ ·6H ₂ O	0.025

^z The author prepares MS medium with monohydrated MnSO₄·H₂O at 16.9 mg l⁻¹, i.e., the same Mn²⁺ 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
1-naphthaleneacetic acid (NAA)	0–0.2
6-benzyladenine (BA)	0.2–10
Gellan gum	2000

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)^z.

Stock solution	Ingredient	Amount (mg)	ml of stock solution per liter of medium		
			Multiplication	Rooting	Hardening
A: Macro 1 ^y	KNO ₃	95,000	20	20	10
	NH ₄ NO ₃	82,500			
	CaCl ₂ .2H ₂ O	22,000			
B: Macro 2 ^y	MgSO ₄ .7H ₂ O	18,500	20	20	10
C: Macro 3 ^y	KH ₂ PO ₄	8500	20	20	10
D: Micro ^x	MnSO ₄ .H ₂ O	1690	1	1	1
	H ₃ BO ₃	620			
	KI	83			
	ZnSO ₄ .4H ₂ O	860			
	Na ₂ MoO ₄ .2H ₂ O	25			
	CuSO ₄ .5H ₂ O	2.5			
	CoCl ₂ .6H ₂ O	2.5			
E: Fe-stock ^w	FeSO ₄ .7H ₂ O	2785	5	5	5
	Na ₂ EDTA.2H ₂ O	3725			
F: Vitamins and amino acids ^x	Glycine	200	1	1	1
	Thiamine.HCl	40			
	Pyridoxin.HCl	50			
	Nicotinic acid	50			
G: Anti-oxidant ^v	Ascorbic acid	1000	1	1	1
H: IAA ^u	IAA	8.75	1	0	0
I: NAA ^u	NAA	9.30	0	1	0
J: BA ⁱ	BA	11.25	20 ^s	1	0

^z All media contain 30 g l⁻¹ sucrose and are solidified with 2 g l⁻¹ gellan gum.

^y Dissolve in distilled water and bring final volume to 1000 ml.

^x Dissolve in distilled water and bring final volume to 100 ml.

^w Dissolve in distilled water by heating to boiling point and bring final volume to 500 ml.

^v Dissolve in distilled water and bring final volume to 50 ml.

^u The auxins IAA and NAA are dissolved in a few drops of ethanol (95%). Bring final volume with distilled water to 50 ml.

ⁱ Dissolve BA in 1 ml 1N NaOH and bring final volume to 50 ml with distilled water.

^s For multiplication medium, it is better to use a more concentrated stock solution prepared by dissolving 112.5 mg BA in 5 ml 1N NaOH and bringing the final volume to 50 ml with distilled water; use 2 ml of this stock solution to prepare 1 liter of multiplication medium.

shoot tips (Krikorian and Cronauer 1984b). However, inclusion of organic additives such as coconut water or casein hydrolysate is not absolutely necessary and, sometimes, even 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) (Mante and Tepper 1983; Banerjee and De Langhe 1985; Vuylsteke and De Langhe 1985; Gupta 1986; F. Novak pers. comm. 1988), in order to reduce blackening of shoot tips (4.1.4 Culture maintenance and 7.1 Blackening of shoot tips).

Plant growth regulators (sometimes erroneously called hormones) are essential media components 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 (1-naphthaleneacetic acid), and IBA (indole-3-butyric acid). BA (6-benzyladenine) is the cytokinin of choice for the induction of shoot-bud proliferation in vitro. BA 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 (4. Micropropagation Methods). The uptake and metabolism of BA in shoot cultures of *Musa* is discussed by Blakesley (1991).

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 addition. However, in the presence of BA, 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 BA and adenine sulphate in promoting shoot development in *Musa textilis* cultures. The inclusion of activated charcoal is discussed below (4.3 Plant establishment).

Important aspects of the culture medium are its solid or liquid state and the pH. A semi-solid medium is more commonly used than a liquid medium. In some laboratories, liquid and semi-solid media are alternated, because the former increases 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.

An elegant liquid culture system, based on temporary immersion of explants with liquid medium (20 min every 2 h) has been described by Alvard et al. (1993). This system may eventually be cheaper by producing more propagules in less time, but it requires special culture vessels and a pressurized pump circuit, thus making it suitable only to the more sophisticated tissue culture laboratory.

Agar was once widely used to solidify media. Its concentration ranged 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 was generally recommended, as it still gave ample firmness to the medium. Agar is no longer the preferred gelling agent, because it contains contaminating compounds and is of inconsistent quality among different batches. Marchal et al. (1992) observed that agar media produced significant amounts of ethylene (a gaseous plant growth regulator that accumulates in closed culture containers and may result in reduced or abnormal plant growth [George and Sherrington 1984]), which is undesirable.

Nowadays, gellan gum (PhytigelTM, Sigma Chemical Co, St Louis, Missouri, USA; or GelriteTM, Merck and Co, Inc, Rahway, NJ, USA) is the most popular gelling agent, because it forms clear gels and is free of contaminating compounds. It also gives more leafy cultures than agar. Many workers have observed that gellan gum gives better results than agar (e.g., Marchal et al. 1992). Gellan gum is generally added at 2 g l⁻¹, i.e., one-quarter of the usual agar concentration.

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 a fresh medium (4.1 Culture initiation). Marchal et al. (1992) reported the same changes in pH during autoclaving and culture.



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.

3.3.2 Media preparation

Media are commonly prepared by dissolving the appropriate chemicals in distilled water, adjusting the pH of the solution, adding gelling agent (for semi-solid media), and autoclaving. MS medium can be obtained from specialist suppliers as packaged preparations consisting 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 (4. Micropropagation 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 ml 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 media, it is advisable also to keep a 10 mM BA stock solution of which 2 ml are added to each liter of medium.

A stepwise procedure for preparing 1 liter of medium is given in Figure 4. All ingredients are added to distilled water and the solution is heated to dissolve the gelling agent. When using agar, care should be taken not to

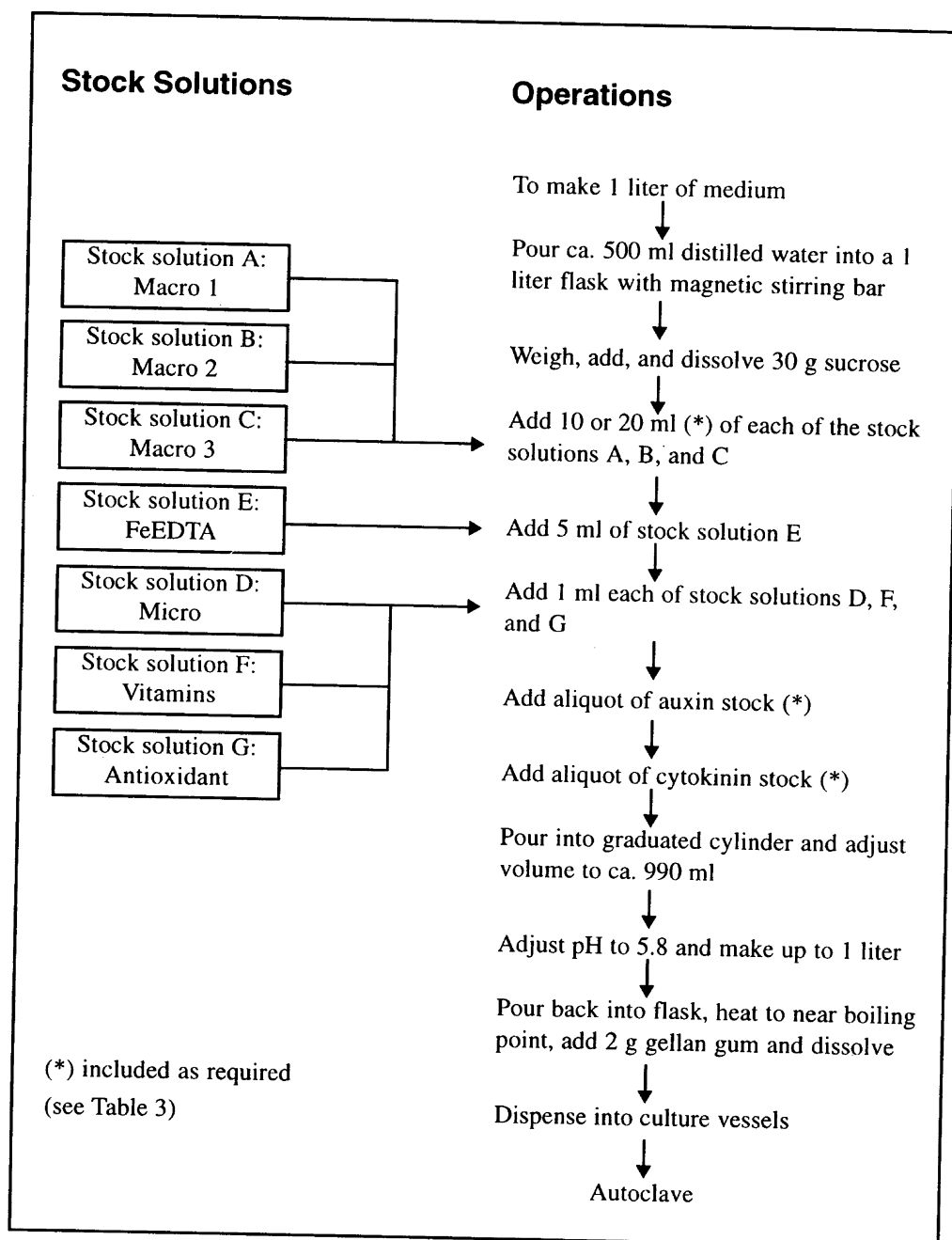


Figure 4. Schematic procedure for media preparation (as adopted for routine practice at IITA).

add it to boiling liquid as the medium is then liable to froth up and boil over. Once the gelling agent has dissolved, aliquots of medium are dispensed (ideally using a dispenser with adjustable volume) into culture vessels, which are then capped. The amount of medium contained per culture vessel varies from 10 to 50 ml (3.4.1 Culture containers).

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 of autoclaving is adequate when media are dispensed into small aliquots, 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 whenever possible as these may induce chemical changes (Bonga 1982). Thiamine and IAA are partly destroyed during autoclaving and can be sterilized by ultrafiltration (George and Sherrington 1984). If the medium is not to be used within 2 days, storage in a refrigerator is preferable (up to 2 weeks). Care should be taken to label and date the containers of media.

3.4 Culture environment

3.4.1 Culture containers

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 many times. Disposable and presterilized plastic containers are becoming more widely available and greatly reduce the amount of routine washing. However, they can be prohibitively expensive.

The volume of the container can sometimes affect in vitro growth. Several workers have 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 × 2.5 cm), containing 20 ml of multiplication medium, for culture initiation and multiplication. The same test tubes can be used for rooting medium (20–30 ml). Alternative containers are petri dishes (10 cm diameter) or wide-necked glass jars (11 × 6 cm). Rooted shoots are usually hardened in jars (50 ml of medium) or petri dishes (30 ml of medium).

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Test tubes can only hold one explant, while petri dishes and jars may contain up to 10 explants each (4.3 Plant establishment). 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 m² can hold about 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 generally maintained in a room with controlled temperature and light regimes (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 airconditioning unit. However, this satisfies the culture requirements of *Musa*.

Most shoot-tip cultures are kept 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 rooting 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, an average light intensity of 50 µmol m⁻² s⁻¹ is achieved at plant tissue level, which is about 2.5% of full sunlight intensity (Côte et al. 1990). Higher light intensities of up to 350 µmol m⁻² s⁻¹ promote more rapid plant growth (Côte et al. 1990; Marchal et al. 1992). Increased light intensity during the last in vitro stage (Stage III, 4.3 Plant establishment) also improves the survival rate of plantlets upon transfer to soil (Murashige 1974; George and Sherrington 1984).

3.5 Nursery area

In vitro plantlets are more fragile than conventional propagules, such as suckers. Successful establishment of these plants thus requires some care and special nursery conditions. Tissue-cultured plants are produced in a closed, sterile environment and grow on artificial nutrient media in controlled conditions. When removed from the tissue culture environment, micropropagated plants must be allowed to adjust to the outside

environment with its varying light levels, changing temperature, reduced humidity, lower nutrient availability, and pathogen presence. This acclimatization of micropropagated plants is achieved during a hardening phase (4.3.3 Nursery establishment of plants). To achieve this step, a nursery area with some facilities for humidity control and partial shading is required.

Various types of nurseries with different degrees of sophistication can be used to harden micropropagated banana/plantain plants. Simple sheds can be made out of locally available materials, such as bamboo or other wooden sticks, roofed with mats of palm leaves or papyrus. Stronger, permanent structures can be constructed from metal pipes in concrete slabs with chainlink and/or shade cloth as cover, and stone chippings on the floor. Specialized greenhouses, screenhouses, or solar domes can be purchased from professional nursery suppliers, but these can be expensive.

Newly transplanted banana plantlets are often kept in a humidity chamber before transfer to normal nursery conditions. Facilities for watering by hosepipe or sprinkler must be available. Partial shading is essential in the early stages of nursery establishment. Shade can be provided by using special shade cloth to cover the nursery or greenhouse frame or by roofing a simple shed with mats of papyrus, palm leaves, or other available plant material. Plants can be exposed steadily to greater sunlight by gradually reducing the shade until full sunlight just before field planting. This can be achieved by progressively moving the shade cloth or mats of leaves.

Various types of nursery containers exist: flats, plastic pots, fiber pots, and polyethylene bags (polybags) of different sizes are suitable. Black polybags are widely used because they are cheap and easy to handle during subsequent field planting. Plants grow best in large 20-liter polybags (30 cm diameter, 30 cm high). Jarret et al. (1985) successfully used such bags. However, they occupy a lot of space and are heavy when handling. Smaller 3-liter polybags (about 15 cm diameter, 20 cm high) are preferred, because they take up less 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 a potting mix. Top soil mixed with composted organic matter is recommended as an improved nursery substrate. Examples are (a) top soil mixed with dry cow manure at a ratio of 12:1, or (b) top soil mixed with

palm fruit pulp and dry chicken manure at 7:2:1. Other potting mixes can be made from locally available materials. 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. The mix should be free draining. Some workers use a 1:1 peat : soil mixture. Potting mixtures containing vermiculite have also been used (Hwang et al. 1984; Krikorian and Cronauer 1984b; Gupta 1986) and can be purchased from horticultural suppliers. A supply of fertilizer helps to maintain rapid growth (4.3.3 Nursery establishment of plants).

The soil or potting mix may be pasteurized or sterilized to keep the plantlets free from soilborne pests and diseases during the nursery stage, but this is not always necessary or feasible. Pasteurization of the soil is usually done by steaming.

4. Micropropagation Methods

The in vitro production of plants proceeds through a sequence of three major steps as defined by Murashige (1974). Stage I: initiation of an aseptic culture; Stage II: multiplication of propagules; and Stage III: rooting of plantlets and establishment in 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 controlled by the medium composition, particularly the growth regulators. Hence, each stage requires a specific 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 propagules can be subdivided and further multiplied by subculturing, or rooted to obtain plantlets (Fig. 5).

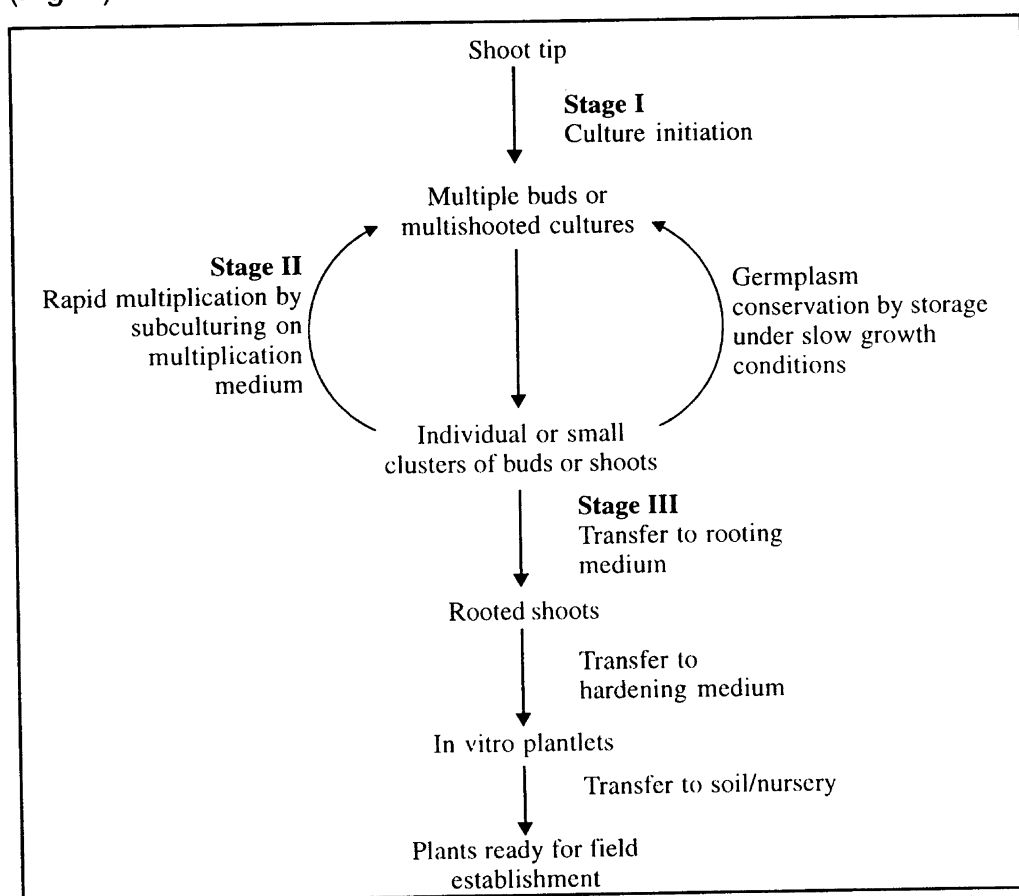


Figure 5. Schematic procedure for the production of banana/plantain plants by shoot-tip culture in vitro.

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 explants.

4.1.1 Disinfection of explants

Shoot tips are preferably harvested from buds, peepers or small sword suckers (3.2 Plant material). Buds or suckers are cut off source plants with a knife or a blade. This plant material is contaminated with microorganisms, 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 that take the form of a set of fitting cones. By virtue of this genuine natural protection against surface contaminants, the apices are easily sterilized. The sterilization procedure outlined below results 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 $2 \times 2 \times 2 \text{ cm}^3$ cube enclosing the shoot apex is obtained (Fig. 6a). Care should be taken to avoid cutting through the apex during this procedure.

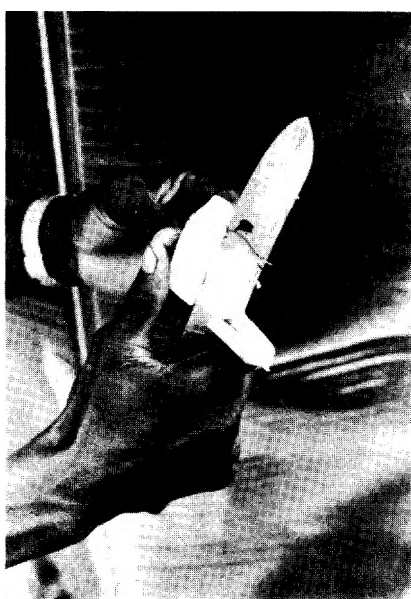


Figure 6a. Disinfection and excision of explants for culture initiation. Trimming of source material to obtain a cubical block of tissue containing the apex.

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.

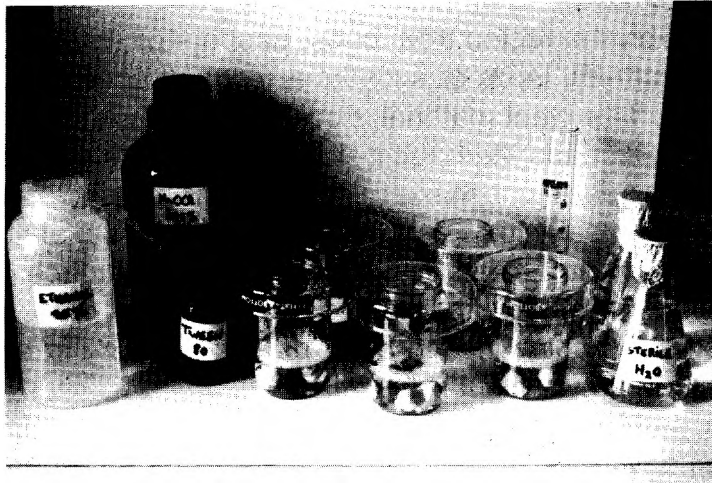
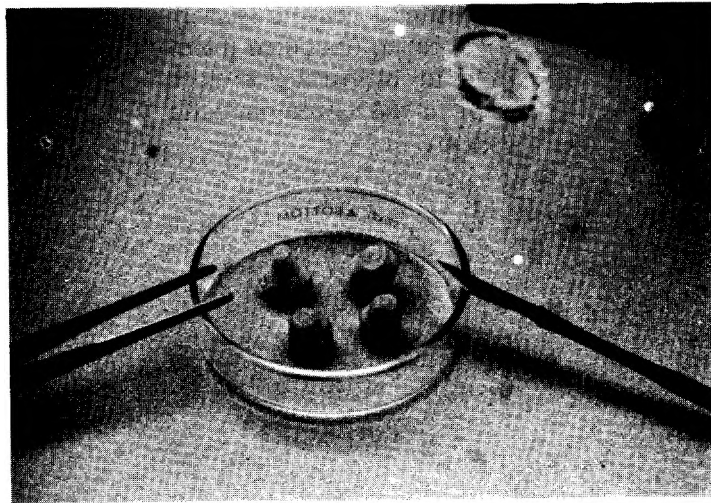


Figure 6b. Disinfection of tissue blocks in a 0.75% sodium hypochlorite solution.

Figure 6c. Surface-sterilized blocks of tissue in a sterile petri dish ready for excision of shoot tips using forceps and scalpel.



3. 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 (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,b; Jarret et al. 1985; Sandoval 1985). [Note: a 1.5% (w/v) calcium hypochlorite ($\text{Ca}(\text{OCl})_2$) 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 given 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 an unnecessarily potent treatment in view of the shoot apex's particular morphology. If the explant is disinfected after excision (Vessey and Rivera 1981; Cronauer and Krikorian 1984a,b), 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, because 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 (Sandoval 1985; Novak et al. 1986). Firstly, larger tissue blocks 5–6 cm in diameter are soaked for 10–40 min in a NaOCl solution 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 extensive disinfection treatment is unnecessary in situations where cultures can be initiated within 24 hours after sucker collection. However, the double sterilization technique is the most effective method in terms of reduced contamination and greater explant survival when 1–2 week-old suckers are used for culture establishment (Hamill et al. 1993). Such situations arise when suckers are collected in remote fields and dispatched over great distances to a central tissue culture laboratory, e.g., during germplasm collecting missions (Sharrock 1995).

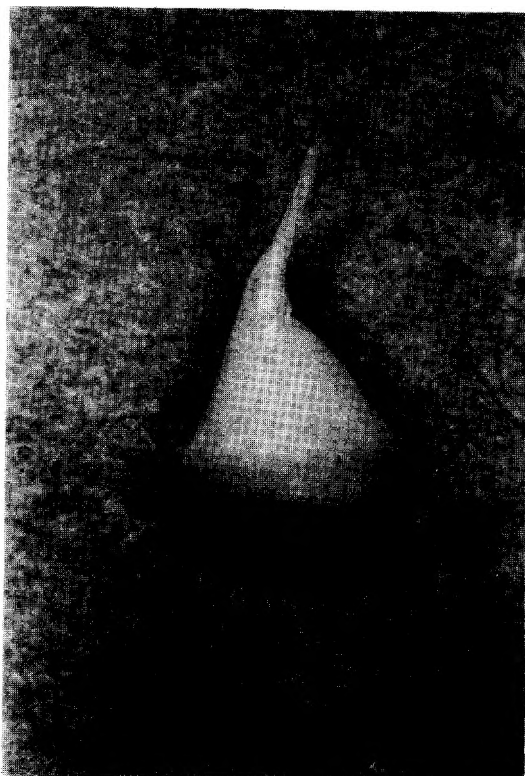
4.1.2 Excision of explants

The next step is the excision of the shoot tip, which requires some 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 of the explant (7.1). To ensure sterile conditions, shoot tips are usually excised in a separate transfer area (3.1.2 Transfer area). Persons handling explants must obviously observe clean procedures and should not lean over the plant material. The operator's hands should be washed with an antiseptic soap followed by spraying with ethanol [70–95% (v/v)]. Sterilized parts of instruments or sterile plant material should not be touched by hands. During manipulations, dissecting instruments are repeatedly soaked in 70–95% ethanol followed by flaming, or sterilized by placing in the electric sterilizer. Instruments should 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. An electric, dry-heat, glass-bead sterilizer is placed next to the rack. Alternatively, a 95% ethanol dip and an alcohol lamp are lined up with the rack holding the instruments for ease of repeated flaming (Fig. 1a,b). 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 disinfected block 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 all sides of the cube.
3. 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. A dissecting microscope or magnifier may be used to facilitate neat excision, but this is not really necessary if one uses relatively large explants (5–10 mm) to initiate cultures.
4. When the shoot tip with its typical conical morphology is reduced to about 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 3–6 leaf primordia and supported on a small base of corm tissue. The excised shoot tip is promptly transferred to a culture container and pushed a short distance into the medium to ensure good contact. Shoot tips are normally placed upright, but explant orientation is not really important.

Figure 6d. Photomicrograph of a freshly excised shoot tip ready for inoculation on to medium (shoot tip is 5 mm long).



Sterilization and excision procedures for floral apices and floral parts are similar to those described above for shoot apices.

Excised shoot tips are often placed directly onto a medium that will encourage shoot-bud multiplication (Table 3), so that Stages I and II are not really discrete. The growth of the shoot tips following culture initiation is, therefore, described in the next section (4.2 Multiplication of propagules). Krikorian and Cronauer (1984b) and many other tissue culture workers 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 media. The cultures are then transferred to multiplication medium 2–3 weeks after initiation (Jarret et al. 1985; Sandoval 1985; Novak et al. 1986).

The shoot apex is sometimes wounded by a series of cuts or fragmented longitudinally just before inoculation onto the medium. This practice, though not always necessary, is described below (4.2.1 Multiple shoot formation in excised shoot tips).

Explants can be pretreated with an antioxidant to reduce blackening of the explant 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 (3.3.1 Media composition; 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 size) increase the likelihood of producing pathogen-free plants, although they have a high mortality rate and grow very slowly (Vuylsteke and De Langhe 1985). Explants that are too large (> 10 mm tall) are also unsatisfactory, because they show more blackening and contamination, and thus lower survival rates than smaller explants (Sandoval and Muller 1992). The latter authors concluded from a study with four banana and plantain cultivars 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 2–5 leaf initials and measuring 3–6 mm in both height and diameter at the leaf base (Krikorian and Cronauer 1984b; Sandoval 1985; Vuylsteke and De Langhe 1985; Gupta 1986; Jarret 1986a,b; Novak et al. 1986). Such explants have shown 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 section 3.4 (Culture environment).

4.1.4 Culture maintenance

Freshly excised shoot tips are creamy white, but the cut surfaces will invariably turn brown or black shortly after excision. 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 gelling agent 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,b). The preferred and definitely the best method to control blackening at culture initiation is to transfer the explants to a 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. In the author's experience, blackening is particularly intense in newly initiated cultures and will decrease with time in culture for many genotypes.

Regular transfer to a 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 (3.3 Culture media) and concentrations of medium components change due to the evaporation of water. Transfer to a fresh medium is recommended 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. Contaminated cultures should be discarded. The problem of contamination is also discussed below (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 BA (3.3.1 Media composition), which is usually added at concentrations of 2–5 mg l⁻¹. BA levels as high as 10 mg l⁻¹ have been used (Dore Swamy et al. 1983; Damasco and Barba 1985), but concentrations above 10 mg l⁻¹ have been found to reduce shoot multiplication (Wong 1986; Zamora et al. 1986). BA at 5 mg l⁻¹ was found to be optimal by Cronauer and Krikorian (1984b), Jarret et al. (1985), F. Novak (pers. comm. 1988),

and Vuylsteke and De Langhe (1985), 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 BA 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:auxin ratio is maintained.

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

Jarret (1986a,b) 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. comm. 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, wounded, or fragmented shoot apex (4.1.2 Excision of explants). The technique of wounding 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.

1. 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.
2. A series of vertical cuts into the meristematic dome were applied by Vessey and Rivera (1981), M. Bustamante (pers. comm. 1983), Jarret et al. (1985), Gupta (1986), and Côte et al. (1990). 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 greatly increase explant and medium blackening (Jarret et al. 1985), but this

may be prevented by the addition of ascorbic acid to the medium (Gupta 1986).

3. 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 (Cronauer and Krikorian 1984b; Krikorian and Cronauer 1984b; Damasco and Barba 1985; Jarret et al. 1985; Sandoval 1985; Novak et al. 1986; Zamora et al. 1986; Epp 1987). Jarret et al. (1985) reported that the method of shoot-tip fragmentation was more effective than the method of apical wounding alone, because it involved less blackening.

Wounding 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 or 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 within 2 weeks. 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 a circular arrangement around the insertion of the inner leaf initial. Sequential removal of the inner 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).

Intact shoot tips are generally left to grow for 6–8 weeks, during which time they may have been transferred to fresh media several times (4.1.4 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 separate, smaller pieces (avoiding transfer of basal corm tissue) on fresh multiplication media. The miniature propagules, 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 clump (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 rooting media as needed. Aseptic conditions must be maintained during all these culture manipulations.

Rates of multiplication of in vitro propagules range from 2 to 10 per month across a range of different *Musa* genotypes (Hwang et al. 1984; Banerjee and De Langhe 1985; Vuylsteke and De Langhe 1985; Côte et

Figure 7a. Highly proliferating shoot/bud cultures that appear as a clump of cormlets each bearing one or more meristems. 1-year-old subculture of *Musa* (ABB) cv. Bluggoe (diameter of tissue clump is 2.5 cm).

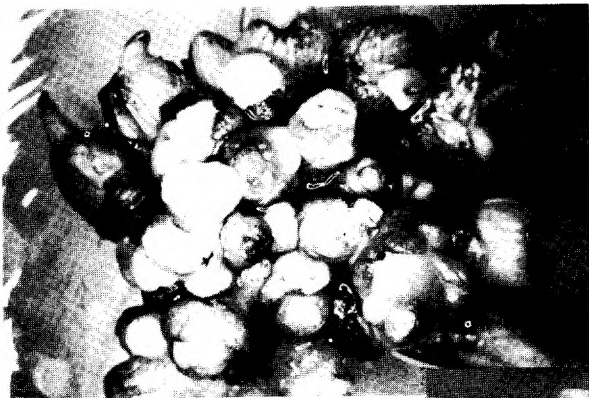
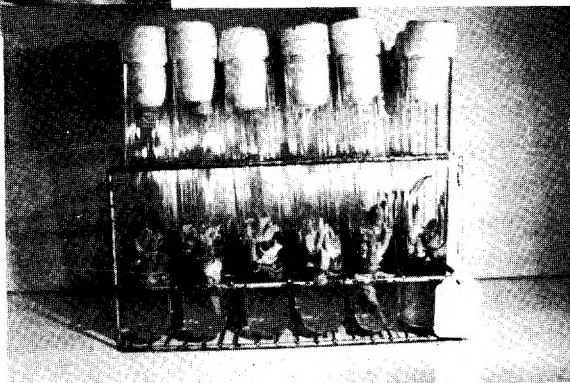


Figure 7b. 2-year-old subculture of *Musa* (AAB plantain) cv. Agbagba.



al. 1990; Talengera et al. 1994). 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 a fresh medium midway through the interval.

Although subculturing can often be continued during many months and even years without adverse effects becoming apparent in culture, off-type shoots could arise through somaclonal variation (Scowcroft 1984; Smith 1988; Israeli et al. 1991; Vuylsteke et al. 1991; Vuylsteke 1998) and be multiplied undetected through several subcultures (7.4 Somaclonal variation).

4.2.3 Factors affecting multiplication rates

Genotype

Many investigators have observed variation among genotypes in the degree and pattern of shoot-bud proliferation in vitro (Table 4). These differences have been observed not only among, but also within the different genomic groups of the *EuMusa* Series of bananas. Presumably, variation in multiplication rate is due to different cultivar-dependent responses to the cytokinin concentration in the medium. 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, as well as Banerjee and Sharma (1988), proposed that the presence of a B-genome in a cultivar's genomic make up could play a role in the achievement of higher proliferation rates. This assumption 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 (*Musa* AAB) and many cooking bananas (*Musa* ABB), appear as clumps of numerous bulbil-like structures or cormlets, each bearing one or several meristem tips on their surface (Fig. 7a). Shoot outgrowth is largely

Table 4. In vitro multiplication rates in shoot-tip cultures of some bananas and plantains (*Musa* spp. L.), showing differences among cultivars. Number of shoots/buds per subculture of 1–2 months on multiplication medium.

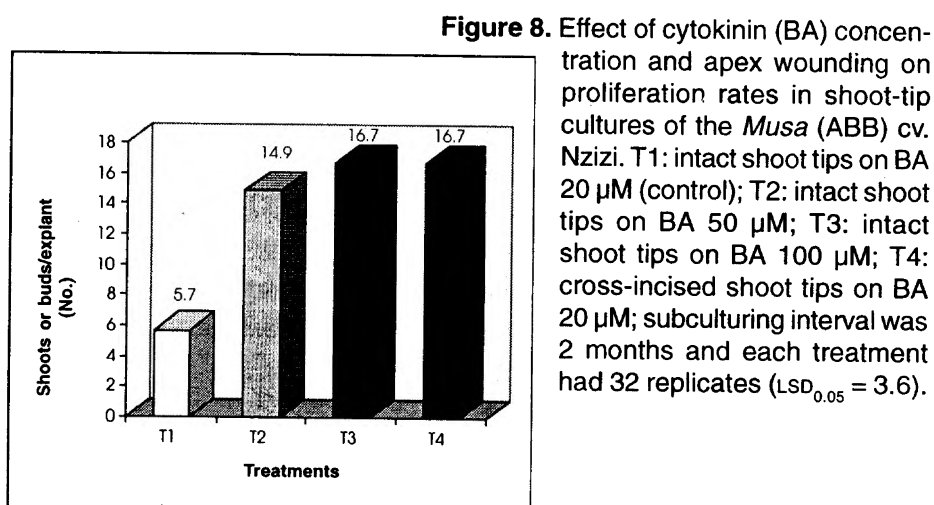
Cultivar and genome	Multiplication rate	Reference
<i>AAA banana</i>		
Gros Michel	2.6	Talengera et al. 1994
Lacatan	2.6	Wong 1986
Mbwazirume	2.8	Talengera et al. 1994
Poyo	5.2	Côte et al. 1990
Valery (10 months) ^z	8.0	Banerjee and Sharma 1988
Dwarf Cavendish	9.4	Banerjee and De Langhe 1985
Valery (14 months) ^z	18.5	Banerjee and Sharma 1988
<i>AAAA banana</i>		
IC2	3.3	Wong 1986
<i>AAB banana</i>		
Mysore	6.6	Wong 1986
Prata	11.7	Banerjee and De Langhe 1985
Silk	16.0	Banerjee and De Langhe 1985
<i>AAB plantain</i>		
Obino l'Ewai	9.2	Vuylsteke et al. 1991
Bobby Tannap	12.4	Vuylsteke et al. 1991
Big Ebanga	15.3	Vuylsteke et al. 1991
Maricongo	16.4	Gupta 1986
Agbagba	16.5	Vuylsteke et al. 1991
Agbagba	19.0	Banerjee and De Langhe 1985
Ubok Iba	20.8	Vuylsteke et al. 1991
Asamiensa	24.4	Banerjee and De Langhe 1985
<i>ABB cooking banana</i>		
Cardaba	3.0	Zamora et al. 1986
Pelipita	18.0	Cronauer and Krikorian 1984b
Bluggoe	20.4	Banerjee and De Langhe 1985
Saba	31.0	Jarret et al. 1985

^z Age of culture

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 shoot/bud proliferation is clearly the cytokinin. Shoot-tip cultures of many banana and plantain cultivars proliferate profusely when cultured on a multiplication medium containing 5 mg l⁻¹ BA. 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 BA concentration above this standard level (Vuylsteke and De Langhe 1985; Zamora et al. 1986; 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 (Cronauer and Krikorian 1984a,b; Zamora et al. 1986; 4.2.1 Multiple shoot formation in excised shoot tips). As shown in Figure 8, both apex incision and increasing cytokinin levels have about the same effect on cultures that do not proliferate well on standard multiplication media. Increasing the BA concentration from 20 µM (control treatment) to 50 µM significantly increased the proliferation rate in 2-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 20 μM BA, produced the same significant increase over the control. Further increasing the BA concentration to 100 μM did not result in any significant improvement on the 50 μM BA 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 their survival rate (Sandoval and Muller 1992). The recommended size of the explant has been discussed earlier (4.1.2 Excision of explants).

For subculturing, it is preferable 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 labor.

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), but this is not consistent among genotypes and laboratories. Banerjee and Sharma (1988) reported that the multiplication and regeneration potential remained unaffected in long-term cultures, even after 30 months. For most cultivars, multiplication rates increased with culture duration.

4.3 Plant establishment (Stage III)

In Stage III, steps are taken to grow rooted plantlets and prepare these for transfer to the external environment. This step is necessary because propagules multiplied during Stage II are very small and not capable of surviving in soil. The main morphogenetic event to take place during this stage is the rooting of shoots. In case mainly buds (Fig. 7a) instead of shoots were produced during the multiplication stage, 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. As such, a separate root-inducing medium has to be used in Stage III. Root initiation in micropropagated shoots, as well as elongation of buds into shoots, is easily accomplished by transferring propagules to the rooting medium given in Table 3 (Fig. 9). 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 or buds each.

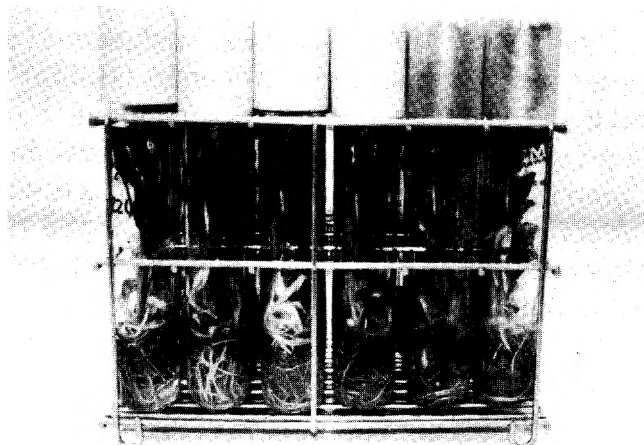


Figure 9. Rooting of micropropagated shoots.
Rooted shoots of micropropagated banana
after 6 weeks on rooting medium.

The cytokinin content of the rooting medium ($1\ \mu\text{M}$ BA) is much lower than in the multiplication medium, so that the cytokinin/auxin ratio becomes ca 1. Most investigators even 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), but the author agrees with Jarret (1986a,b) in recommending the inclusion of auxin.

The auxins most frequently incorporated into media to induce rooting are NAA, IAA, or IBA. The author found that NAA ($1\ \mu\text{M}$ or approx. $0.2\ \text{mg l}^{-1}$) was more effective than IAA in promoting 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, while IAA gave less than 50% success. Higher NAA concentrations (2 and $5\ \mu\text{M}$) did not give better results than $1\ \mu\text{M}$, but Mante and Tepper (1983) and Cronauer and Krikorian (1984b) found 1

mg l⁻¹ (ca 5 µM) to be more satisfactory in *Musa textilis* and AAA and ABB bananas, respectively. Because NAA (0.2–1 mg l⁻¹) readily induces rooting in many banana and plantain cultivars, it is the preferred auxin for the rooting medium.

IBA was also found to be effective at 1 µM (Banerjee and De Langhe 1985; 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; Sun 1985). However, inclusion of activated charcoal is not necessary; Cronauer and Krikorian (1984b) and Jarret et al. (1985) found that it failed to promote root initiation.

Satisfactory rooting can occur on a full strength MS medium, but in some laboratories it is common practice to induce rooting on a medium with macronutrients at half concentration (Vuylsteke and De Langhe 1985; Novak et al. 1986). Mante and Tepper (1983) and F. Novak (pers. comm. 1988) also reduced the sucrose content to 1–1.5%.

Typically, white cord roots appear at 4–14 days after transfer to the rooting medium if the Stage II propagules are leafy shoots of at least 1 cm tall. However, roots can take up to 1 month to appear. It is clear that if the explants consist of bud-like structures (Fig. 7a), 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 (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 (4.3.2 In vitro hardening of planlets) prior to transplantation to soil.
2. The rooted shoots can be left for 2–3 weeks on the rooting medium and are then transferred directly to the external environment (4.3.3 Nursery 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 behavior 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.

4.3.2 *In vitro* hardening of plantlets

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

Figure 10a. *In vitro* hardening of micropropagated plantain plantlets. 4 weeks in a petri dish (diameter is 10 cm).



Figure 10b. In glass jar (11 cm high).

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 a rooting 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 a rooting medium for 4–6 weeks. Such plantlets show survival rates exceeding 90% when transplanted directly to the soil.

Murali and Duncan (1995) found that incorporation of triadimefon (1–2 mg l⁻¹, a triazole fungicide used in black sigatoka control, which appears to also have plant growth regulating effects) in the Stage III culture medium significantly improved the hardiness and survivability of micropropagated banana plantlets upon transplantation from in vitro conditions to the nursery. However, the author is unaware of widespread application of this medium addition for this purpose.

4.3.3 Nursery 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 significant losses of plants if not done carefully. Micropropagated plants are delicate propagules because they are produced in a closed, sterile environment and grown on nutrient-rich artificial media under controlled conditions with high humidity and low light intensity. When removed from the tissue culture environment, micropropagated plants must be allowed to adjust to the outside environment with its varying light levels, changing temperature, reduced humidity, lower nutrient availability, and pathogen presence. Tissue-cultured plants may lose water rapidly upon transfer to natural conditions. Moreover, they are believed to have limited photoautotrophic capacity, so that their energy demands must initially be met by reserves of starch accumulated during culture.

Rooted shoots that are 7–10 cm tall and have a strong root system are ready to be transplanted into a potting mixture. As indicated earlier (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 medium is gently washed from the roots. If a cluster of plantlets is produced at the end of Stage III (Fig. 11a), these 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 potting mixture, taking care not to damage the fragile roots. The mixture should cover the upper roots by 1–2 cm. After transplanting, plantlets are immediately watered. Although the potting mixture must be kept moist initially, there is a narrow line between too much and too little water. Jarret et al. (1985) argue that control of soil moisture is a critical factor in successful plantlet establishment. Maintenance of high humidity is also important and can be achieved by intermittent misting or by keeping the plantlets under a relatively low plastic cover in a humidity chamber. Bower and Fraser (1982) covered plantlets individually with transparent polyethylene bags. Plantlets should also be kept in partial shade and progressively hardened by gradually reducing the humidity and shade. When these precautions are observed, survival rates in the nursery should be 90–100%. It is beneficial to apply a dilute solution of fertilizer 4–6 weeks after transplantation (Fig. 11b). Urea (0.1–0.5 g) and potash (KCl at 0.2–1 g), dissolved in 100 ml of water 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) until they are 20–30 cm tall. Field preparation, planting, and



Figure 11a. Nursery establishment of in vitro micropropagated plantlets. Clusters of plantlets ready for pot transplantation after separation into individual plantlets.

Figure 11b. Young plantain plantlets 1 month after transplantation (soil is contained in 3.5 l polybags).



crop management should be performed according to recommended practices (Stover and Simmonds 1987; Tezenas du Montcel 1987; Swennen 1990) to achieve successful field establishment and subsequent vigorous growth (Fig. 12b). Separate manuals on the postflask management of micropropagated *Musa* are available from Daniells and Smith (1991) and Vuylsteke and Talengera (1998).

4.3.4 Field performance of micropropagated plants

Vuylsteke (1998) recently reviewed reports on the field performance of micropropagated bananas and plantains. Most reports concern micropropagated bananas of the Cavendish subgroup (*Musa* spp., AAA group), which constitute the bulk of the international banana trade (Stover and Simmonds 1987). When compared with bananas propagated by conventional planting material, i.e., suckers, micropropagated banana plants were capable of performing equally or better (Hwang et al. 1984; Smith and Drew 1990a). In general, micropropagated banana plants established more quickly, grew more vigorously, were taller, had a shorter and a more uniform production period, and produced higher yields than conventional propagules (Hwang et al. 1984; Daniells 1988;



Figure 12a. Establishment in soil of micropropagated plants. Plantain plants, 2.5 months in the nursery (in 20-liter polybags), ready for field planting.



Figure 12b. Micropropagated plantain plants, 3 months after field planting.

Israeli et al. 1988; Zamora et al. 1989; Drew and Smith 1990; Kwa and Ganry 1990; Espino et al. 1992; Robinson et al. 1993). Yield gains from the use of in vitro plants reached up to 20% (Table 5), but this was sometimes confined to the plant crop only or was season-dependent in the subtropics (Drew and Smith 1990; Israeli et al. 1988). Generally, superior performance of micropropagated bananas was due to the fact that such propagules already possessed an active root and shoot system at the time of planting, and were free of most diseases and pests (Drew and Smith 1990).

Table 5. Yield gain of micropropagated over conventional propagules of banana and plantain (*Musa* spp. L.).

Cultivar	Yield gain (%)	Country	Reference
<i>Cavendish bananas (AAA)</i>			
Williams, Grand Nain	20**	South Africa	Robinson et al. 1993
New Guinea Cavendish	16**	Australia	Drew and Smith 1990
Unspecified	12 ^{NA}	Côte d'Ivoire	Kwa and Ganry 1990
Williams	10 ^{NA}	Israel (summer)	Israeli et al. 1988
Williams	7**	Australia	Daniells 1988
Giant Cavendish	2 ^{NS}	Taiwan	Hwang et al. 1984
Grand Naine	-18 ^{NA}	Israel (spring)	Israeli et al. 1988
<i>Plantains (AAB)</i>			
Congo Enano	74**	Puerto Rico	Liu et al. 1989
Maricongo	30 ^{NS}	Puerto Rico	Liu et al. 1989
Enano Comun	16 ^{NS}	Puerto Rico	Liu et al. 1989
Congo	13 ^{NS}	Puerto Rico	Liu et al. 1989
Agbagba	6 ^{NS}	Nigeria	Vuylsteke and Ortiz 1996
<i>Cooking banana (ABB)</i>			
Cardaba	12 ^{NS}	Philippines	Espino et al. 1992

NS, *, **, Not significant or significant at $P \leq 0.05$ or 0.01, respectively; ^{NA}, mean separation not available.

Field performance of micropropagated vs. conventionally propagated plantains (*Musa* spp., AAB group) has been reported in only two studies (Liu et al. 1989; Vuylsteke and Ortiz 1996). In both cases, micropropagated plantains grew vigorously and were taller than conventional plants. However, this vigor resulted in significant yield increases of 13–74 % only in Puerto Rico (Liu et al. 1989), while Vuylsteke and Ortiz (1996) did not observe the expected higher yield due to severe black sigatoka disease and suboptimal husbandry (Table 5).

Results of bananas in Israel (spring planting; Israeli et al. 1988) and plantains in Nigeria (Vuylsteke and Ortiz 1996) suggest that micropropagated plants of *Musa* do not manifest consistently superior horticultural performance compared to sucker-propagated plants. It seems that in vitro plants should be regarded as one component of good crop husbandry, not as a guarantee to achieve maximum yields. Micropropagated plants, in combination with other good cultural practices,

such as correct planting time (particularly in the subtropics), fertilizer or manure, and mulch, will establish quickly, have vigorous vegetative growth, and produce high yields of good quality fruit (Robinson et al. 1993; Vuylsteke and Ortiz 1996). Furthermore, the improved phytosanitary status of in vitro plants makes them the planting material of choice to reduce the risk of pest and disease introduction in new plantings (Hwang et al. 1984; Robinson 1996) or for establishment of field nursery areas (Smith and Drew 1990a). These nurseries can then provide clean conventional propagation material for production fields. Roguing of somaclonal variants could also be performed in such nurseries to avoid the occurrence of off-types in production fields at great economic loss to the plantain or banana farmer.

Tissue culture plants thus have great potential to augment the positive effects of other inputs, particularly in high-input agriculture. However, a troublesome characteristic of micropropagated bananas is their increased susceptibility to virus, fusarium wilt, and nematodes, when compared to conventional propagules. Several authors reported that cucumber mosaic virus incidence was greater in micropropagated than in conventional bananas (Israeli et al. 1995; Kwa and Ganry 1990; Sarah et al. 1990). Smith et al. (1997) observed that micropropagated plants of the susceptible Williams and resistant Goldfinger showed significantly higher incidence and severity of fusarium wilt (race 4) infection than plants grown from conventional planting material. The greater pest/disease susceptibility of in vitro plants generally disappears as the crop ratoons. The cause and mechanism of this phenomenon are not well understood.

5. In Vitro Conservation

Germplasm conservation in field gene banks of a vegetatively propagated crop like *Musa* is fraught with many problems (De Langhe 1984). The use of shoot-tip culture techniques in maintaining such collections, particularly for safety duplication purposes,

has considerable advantages: in vitro cultures occupy a relatively small amount of space, are suitable for international exchange if disease-free, and are amenable to rapid multiplication when required. A disadvantage is that in vitro methods can result in the loss of genetic integrity of cultured material (7.4 Somaclonal variation).

The simplest approach to in vitro germplasm storage would be to follow the procedure outlined above (4.2.2), in which multiple shoot cultures are maintained under normal conditions and subcultured every 2 months (Zamora et al. 1986). However, such a procedure entails frequent transfers, which is labor intensive, time consuming, and increases the risk of losses due to culture contamination. Two more appropriate approaches are available for the conservation of in vitro cultures, which preclude the need for frequent manipulations: slow growth for medium-term storage and cryopreservation for long-term storage.

5.1 Cryopreservation

Although research on the cryopreservation of *Musa* tissue cultures started in the mid-1980s, the focus has been on the use of cell suspension cultures (Panis et al. 1990), because such cultures were less damaged by ice during the cryopreservation procedure. However, the procedures for the initiation of cell suspension cultures, and the regeneration of plants from them, remain tedious and genotype specific (Dhed'a et al. 1991).

Recently, a simple technique was developed for the cryopreservation of banana meristem cultures (Panis et al. 1996). The method involves 2–4 weeks preculture of proliferating meristems on a high sucrose (0.3–0.5 M) medium. Surviving meristematic clumps are then excised, transferred to cryotubes, and plunged into liquid nitrogen for rapid freezing. This procedure was tested on seven banana and plantain cultivars of different ploidy and genomic constitution and found to result in postfreezing viability rates of 12–72%. This simple method should greatly facilitate the long-term conservation of *Musa* genetic resources, because it is based on the widely applicable shoot-tip culture technique.

5.2 Slow growth

Storage of *Musa* shoot-tip cultures under slow growth conditions has been achieved successfully since the early 1980s. It is the method currently used at the *Musa* Germplasm Transit Center of the International Network for the Improvement of Banana and Plantain (INIBAP), which holds the largest in vitro collection of *Musa* germplasm in the world (Van den houwe and Jones 1994).

Methods to reduce the growth rate of in vitro shoot-tip cultures involve modification of either the culture medium or of the physical environment in which cultures are incubated. The latter method, entailing reduced temperature and light, seems to be the preferred approach. Banerjee and De Langhe (1985) maintained proliferating cultures of seven cultivars for more than a year under conditions of low temperature (15 °C) and low light (about one-third of normal intensity). Slow-growth cultures were kept on a medium containing 10 µM BA. Jarret et al. (1986) stored 38 *Musa* accessions for up to 18 months at 18 °C and low light intensity. Their procedure is similar to that of Banerjee and De Langhe, but cultures were maintained on filter paper bridges in liquid media. Núñez et al. (1992) also kept shoot-tip cultures of several cultivars at temperatures ranging from 14° to 20 °C for several months. Ko et al. (1991) found that the storage period of shoot cultures could be extended to 24 months without loss of viability by growing cultures on a 3% ribose solution instead of on normal media.

Van den houwe et al. (1995) reported on the extensive experience in medium-term conservation of *Musa* germplasm at the INIBAP Transit Center. The average storage duration of shoot cultures of 401 banana clones conserved under slow growth conditions of reduced temperature and light (16 °C, 25 µmol m⁻² s⁻¹) was 11 months. However, large differences in storage potential were observed among the genotypes, with some requiring subculturing every 2 months and others keeping for about 20 months. Bhat and Chandel (1993), while confirming that low-temperature incubation is effective for in vitro conservation, however, argued that low temperature storage is not promising, mainly because of the high running costs and the risk of equipment failure, particularly in developing countries. These authors maintained shoot cultures of more than 100 cultivars at 25 °C for 10–12 months without recourse to any growth inhibitory treatment.

An alternative, but less common approach to slow growth, 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 μ M). Mora et al. (1986) achieved a 50% growth reduction by supplementing the medium with 4% mannitol and 3–9% sucrose. Bhat and Chandel (1993) also found that mannitol (2–4%) enhanced the storage period by 2–3 months, but had deleterious side effects at higher concentrations.

Storage of *Musa* germplasm in slow to normal growth conditions for up to 18 months is feasible (Jarret et al. 1986; Bhat and Chandel 1993; Van den houwe et al. 1995). Cultures should nevertheless be checked for contamination or necrosis at regular intervals, and discarded or transferred to fresh media as necessary.

6. Germplasm Distribution

Shoot-tip cultures are very suitable material for the local and international movement of *Musa* germplasm. In fact, they are now generally considered as the only acceptable material for the international exchange of banana and plantain germplasm (Vuylsteke

et al. 1990a; Diekmann and Putter 1996). Shoot cultures, in combination with third-country quarantine (Vuylsteke et al. 1990a) or preferably with virus indexing procedures (Diekmann and Putter 1996), have been used as a vehicle for the safe exchange of *Musa* germplasm since the early 1980s. The advantages of this method include the reduced volume and weight of in vitro cultures and the improved phytosanitary status of germplasm. Viruses are not effectively eliminated by tissue culture (Drew et al. 1989; Wu and Su 1991), hence virus testing of germplasm now is recommended as a routine procedure to ensure its safe international distribution. Reliable virus diagnostic techniques are being developed for the major characterized *Musa* viruses.

6.1 Technical guidelines

The FAO/IPGRI Technical Guidelines for the Safe Movement of *Musa* Germplasm (Diekmann and Putter 1996) recommend the following procedures for vegetative material:

- Select a sucker from a plant without symptoms of systemic infection.
- The sucker should be trimmed to remove soil, roots, and any other extraneous material, leaving part of the central corm containing the meristem and about 10 cm above it. The overall dimension of the block of tissue will be about 20 cm high and 10–15 cm in diameter. The block should be air-dried for 2–3 days and wrapped in newspaper. The material should be labeled and dispatched in a cardboard box. No plastic should be used for wrapping.
- The material should be sent to an appropriate tissue culture laboratory in the country of origin, or if this is not possible, a tissue culture laboratory which preferably should not be in a banana-growing area.
- Meristems should be excised, surface-disinfected, and cultured.
- The meristem culture should be cloned to seven plantlets, of which five should be sent to an indexing facility and two should remain in culture for future multiplication.
- At the indexing facility, four plants should be established in a vector-

free, insect-proof greenhouse under conditions conducive to vigorous plant growth (the fifth serves as a back-up).

- After 3 months of growth, tissue samples should be taken from the three youngest expanded leaves and indexed for viruses as described by Diekmann and Putter (1996).
- Three months later, tissue samples should again be taken from the three youngest expanded leaves and indexed for viruses as described by Diekmann and Putter (1996). In addition, electron microscopic observations should be undertaken to look for the presence of other viruses.
- If all tests are negative, the four indexed plants may be released and the cultures derived from the two remaining subclones may be further propagated and distributed in vitro. For the movement of in vitro material, neither charcoal, fungicides nor antibiotics should be added to the medium. In vitro cultures should be shipped in transparent tubes and visually inspected for bacteria, fungi, and arthropods. Contaminated material should be destroyed.

Presently, one transit center, which also maintains a large in vitro collection, is operated by INIBAP at the Laboratory of Tropical Crop Improvement, Katholieke Universiteit Leuven, Heverlee, Belgium. More information can be obtained from this center. Three indexing centers are listed in the FAO/IPGRI guidelines; these are the Virology Research Units at the Department of Primary Industries, Queensland, Brisbane, Australia; the Taiwan Banana Research Institute, Pingtung, Taiwan; and CIRAD-FLHOR, Montpellier, France. The Virology Research Unit at IITA, Ibadan, Nigeria, can also index *Musa* germplasm for viruses.

6.2 Practical guidelines

The following steps should be taken to achieve successful shipment of shoot-tip cultures:

- The type of culture (proliferating or rooted shoots) to be shipped should take 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 (4.2 Multiplication of propagules). In the absence of such a facility, rooted shoots (4.3 Plant establishment) that do not require any further aseptic handling should be sent.

- The use of glass culture vessels should be avoided as they are liable to break. Transparent plastic culture tubes with tightly fitting screwcaps or plastic petri dishes have given good results. Special tissue culture bags that are heat sealed are available from commercial tissue culture suppliers and are easy to use.
- Cultures to be shipped must be free of bacterial, fungal, or viral infection. For details about virus indexing, refer to the FAO/IPGRI guidelines (Diekmann and Putter 1996).
- For international shipment, arrangements should be made with the quarantine officials who must issue a phytosanitary certificate. An import permit from the quarantine service of the receiving country is also needed.
- 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).
- 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 media with an increased concentration of gelling agent (double the normal concentration) and contained in the smallest possible 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. Inter-continental shipments of *Musa* germplasm have also been lost due to contamination caused by fluctuating pressures in transit (J. Schoofs pers. comm. 1988).
- Each shipment should include a complete list of 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 researchers have reported successful international movement of *Musa* germplasm in the form of shoot cultures (Jarret et al. 1985; Zamora et al. 1986; Vuylsteke et al., 1990a,b). 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 biological problems can be encountered during the course of the in vitro culture procedure or when recovering plants from culture: blackening of explants, contamination of cultures, culture deterioration, and somaclonal variation.

7.1 Blackening of explants

Explants and media commonly turn brown or black during culture initiation and, to a lesser extent, during subculturing (4.1.4 Culture maintenance). Blackening is caused by oxidation of phenolic compounds in wounded banana tissues, and will first appear on the cut surfaces of freshly excised shoot tips and subdivided shoot/bud 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 one or a combination of the three treatments mentioned earlier (4.1.4 Culture maintenance) be applied. This is labor intensive, but necessary to keep valuable germplasm 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 onto the medium.
2. Include antioxidants in the culture medium (3.3.1 Media composition). 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 cultures to fresh media. When blackening is severe, cultures should be transferred to a fresh medium 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 genotypes prone to blackening on media with BA 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 (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 wide mouths, are more liable to become infected during improper culture manipulations. Furthermore, because such 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 meristem microdissection, by including antibiotics in the culture medium, or by bleaching and reinitiation of the cultures.

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 by hypochlorite (4.1.1 Disinfection of explants), and shoot tips excised from them can be 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.

Van den houwe et al. (1998) found 4.5% of the INIBAP Transit Center gene bank to be infected with endogenous bacteria, most of which were identified as *Bacillus* spp. These bacteria were not readily apparent in culture, nor did they negatively affect the storage, multiplication, or rooting of cultures, but "haloes" of bacterial growth occasionally formed around the shoot-tip base. Using a simple, cheap, and nondestructive detection method, which consists of streaking the explant base onto a bacteriological medium, contaminated cultures were detected and eliminated. Therapeutic treatment was only used when an entire accession was found to be contaminated. Excision and reculture of meristem tips of only 1 mm in size, isolated from contaminated cultures, proved 50–100% effective for

the elimination of bacteria. This technique was easy, but resulted in relatively high mortality (33%) of explants due to their small size (4.1.2). Alternatively, culture of contaminated shoot tips for 1 month in a liquid medium with the antibiotic rifampicin (100 mg l^{-1} , filter-sterilized) resulted in 100% recovery of bacteria-free explants without phytotoxicity.

7.3 Culture deterioration

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

7.4 Somaclonal variation

One of the most crucial aspects of using 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 culture, appears to be widespread (Scowcroft 1984, 1985). Somaclonal variation is ubiquitous in *Musa*, as in other plants. *Musa* plants produced by shoot-tip culture have shown somaclonal variation rates of 0–70%, mainly depending on the genotype (Table 6) (Smith 1988; Vuylsteke et al. 1991; Israeli et al. 1995). This potential heterogeneity has often been acknowledged as a problem or risk associated with the use of in vitro culture techniques for germplasm handling. Conversely, somaclonal variation has generated interest as a potential source of novel and useful variability (Vuylsteke 1998).

The range of somaclonal variants recovered through shoot-tip culture is narrow and mostly mimics naturally occurring variation or produces defective phenotypes (Smith 1988; Israeli et al. 1991, 1995; Vuylsteke et al. 1991; Côte et al. 1993). The most commonly observed phenotypic changes are dwarfism in the Cavendish bananas and inflorescence variation in the plantains. Changes in leaf, pseudostem and flower sizes, shapes, colors, and orientations are also common. With respect to their field performance, variants are mostly inferior to the original clone from which they were derived, in that bunch and fruit of variants are often smaller (Smith and Drew 1990b; Vuylsteke et al. 1996). Reduced fruit size and quality offset the potential benefits of variants with putative disease resistance.

The rate of somaclonal variation is mainly influenced by genotype and initial explant characteristics, and not by length of time in culture, rate of multiplication, or medium composition (Vuylsteke et al. 1991; Krikorian et al. 1993; Reuveni et al. 1993; Israeli et al. 1995).

Measures to minimize variation have been recommended by several workers (Smith 1988; Reuveni and Israeli 1990; Côte et al. 1993; Israeli et al. 1995; Robinson 1996) and can be summarized as follows:

- Vigorous sword suckers from superior and true-to-type mother plants should be selected as sources of primary explants.
- Subculturing and multiplication should be limited to 10 cycles (not exceeding 1 year of subculturing) or only 1000 plants per primary explant.
- Plants should be screened in the nursery for early detection of variants using morphological characteristics or other detection methods. Off-types should be rogued. Early detection methods for particular off-types have been described by Smith and Hamill (1993) and Damasco et al. (1996a,b).
- If possible, only stable parent clones and explants should be selected and used for in vitro micropropagation.

Table 6. Nature and frequency of somaclonal variation in micropropagated bananas and plantains (*Musa* spp. L.).

Cultivar and genome	Variation frequency (%)	Type of variation	Reference
<i>Cavendish banana (AAA)</i>			
Grand Nain	1.1	Dwarfism, gigantism Abnormal foliage	Arias and Valverde 1987, 1988
Grand Nain, Williams	1.8	Dwarfism	Robinson et al. 1993
Giant Cavendish	2.4	Dwarfism	Hwang 1986; Hwang and Ko 1987
New Guinea Cavendish	3.0	Abnormal foliage	Drew and Smith 1990
Williams	5.2	Dwarfism	Reuveni et al. 1993
Williams	15.0	Abnormal foliage	Reuveni and Israeli 1990
Williams	20.8	Dwarfism	Smith 1988
Grande Naine	25.0	Abnormal foliage	Stover 1987
Williams	50.0	Dwarfism	Daniells 1988
<i>Plantain (AAB)</i>			
Bobby Tannap	0	None	Vuylsteke et al. 1991
Mbi Egome 1	0	None	Vuylsteke et al. 1997
Ntanga 2	0.4	Plant stature	Vuylsteke et al. 1991, 1997
Obino l'Ewai	3.0	Bunch orientation Stem, bract color	Vuylsteke et al. 1991, 1997
Ihitisim	3.8	Abnormal foliage	Vuylsteke et al. 1997
Agbagba	5.4	Inflorescence variation	Vuylsteke et al. 1991, 1997
Maricongo	7.0	Inflorescence variation	Krikorian et al. 1993
Falso Cuerno	10.8	Inflorescence variation	Sandoval et al. 1991
Ubok Iba	11.0	Dwarfism, gigantism Inflorescence variation petiole color	Vuylsteke et al. 1991, 1997
Maricongo	21.0	Inflorescence variation	Ramcharan et al. 1987
Big Ebanga	25.6	Inflorescence variation	Vuylsteke et al. 1991, 1997
Dwarf Horn	38.0	Inflorescence variation	Ramcharan et al. 1987
Bise Egome 2	74.7	Inflorescence variation	Vuylsteke et al. 1991, 1997
<i>Cooking banana (ABB)</i>			
Saba	0	None	Stover 1987

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