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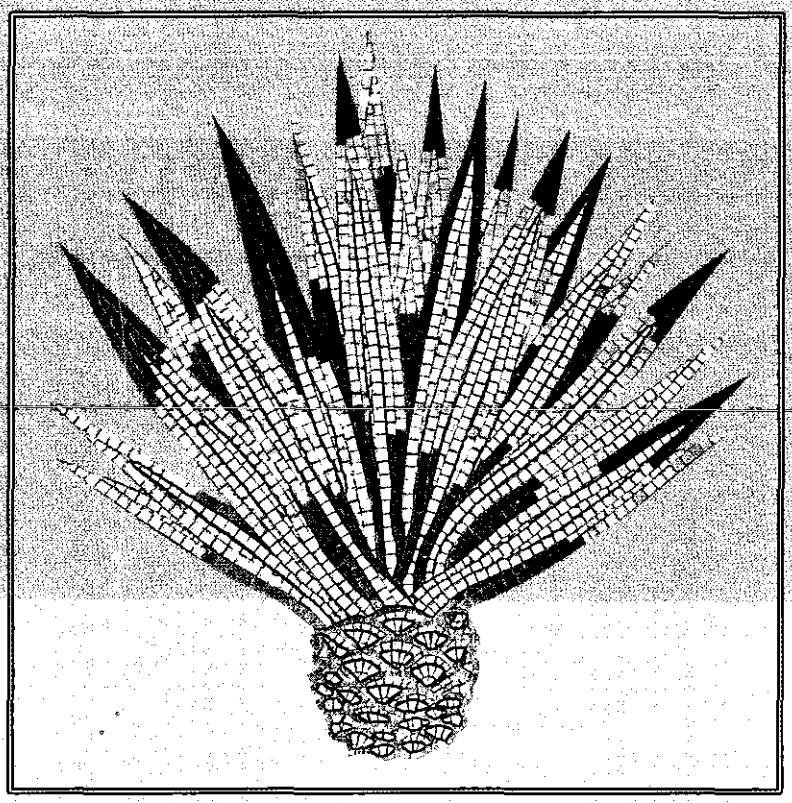
Common Fund for Commodities
Technical Paper no. 38

MANUAL FOR THE *IN VITRO* CULTURE OF AGAVES



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Manual for the i*n Vitro* Culture of Agaves

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION

Vienna 2004

First published October 2004

This Manual has been produced by a team of experts in the framework of a Common Fund project ***(CFC/FIGHF/07 – Product and Market Development of Sisal and Henequen Products)*** financed at the request of the Food and Agriculture Organization of the United Nations (FAO) Intergovernmental Group on Hard Fibres (FIGHF). The project was implemented by the United Nations Industrial Development Organization (UNIDO) under the supervision of the FIGHF.

The **Manual for the *in Vitro* Culture of Agaves** is the second document resulting from the project which is included in the CFC Technical Paper series. The first publication was entitled: **SISAL – Past Research Results and Present Production Practices in East Africa** (CFC Technical Paper no. 8).

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Manual for the i*n Vitro* Culture of Agaves

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Foreword

Like many other natural fibres, sisal is facing strong competition from synthetic materials. In order to retain its current market share and to re-gain its lost markets, the sisal industry needs to increase both its production efficiency as well as to develop new applications and open up new markets. The Common Fund for Commodities is providing support to the sisal sector, through its financing of several projects, to attain the same.

The Fund-supported project "Product and Market Development of Sisal and Henequen Products" (CFC/FIGHF/07) has been implemented by the United Nations Industrial Development Organization in Kenya and Tanzania during the years 1997 - 2004. The designated international commodity body for hard fibres, the FAO Intergovernmental Group on Hard Fibres (FIGHF), supervised its implementation.

One of the components of this project focused on the improvement of the vegetative material which is the basis of the quality of the sisal plants. The emphasis was both on developing a reliable method of producing high quality, disease free plantlets as well as by-passing the costly and time consuming stage of propagation through bulbils in designated nurseries. The method of Meristematic Tissue Culture (MTC) was used in this project in sisal laboratories in both Kenya and Tanzania, with the objective of multiplying promising sisal cultivars to promote the production of standard planting materials and to shorten the time needed by breeders to produce new varieties for commercial production. In order to document the MTC practices developed and used in the project, the current Manual was prepared by the project.

It is hoped that the Manual will provide insights in the techniques and methods applied and serve as a reference document for researchers in the field of sisal (re-) production.

Amb. Ali Mchumo
Managing Director
Common Fund for Commodities

Contents

Acronyms / Abbreviations

ABA	Abscisic Acid
AFLP	Amplified Fragment Length Polymorphism
ARI	Agricultural Research Institute
BAP	6-Benzyl Aminopurine (cytokinin)
Bio-MINT	Modular temporary immersion bioreactor
B5	Culture media
CAM	Crassulacean Acid Metabolism
CFC	Common Fund for Commodities
CICY	Centro de Investigación Científica de Yucatán
2,4-D	2, 4 dichloro Phenoxyacetic Acid (auxin)
IAA	Indol-3-Acetic Acid (auxin)
IBA	Indole Butyric Acid (auxin)
KEPHIS	Kenya Plant Health Inspectorate services
µM	*Micro mole*
mM	Mili mole
MS	Murashige and Skoog Culture Media
MS-B	Modified Murashige and Skoog Culture Media
NAA	Naphtalene Acetic Acid (auxin)
PCR	Polymerase chain Reaction
PPM	Plant Preservative Mixture
SH	Schenk and Hildebrandt Culture Media
UNIDO	United Nations Industrial Development Organization
USD	United States dollars

Preface

In order to promote new alternatives for the hard fibre producing agaves in East Africa, the Common Fund for Commodities (CFC) has, over the past seven years, financed the project titled: "Product and Market Development of Sisal and Henequen Products". Its main objectives have been the development of new end uses for sisal and henequen and the definition of a strategy for their marketing.

To achieve these aims the project contemplated the improvement of productivity in the field and in the processing techniques, particularly those used for the production of pulp paper. An important part of the strategy has included the development and introduction of new sisal varieties for which the use of modern biotechnologies is indispensable. In this context, meristem culture was a much needed technique in order to reduce the breeding period and speed up the introduction of the new varieties through micropropagation.

Meristem tissue culture was included as sub-component A5 of the project with the objective of establishing working tissue culture laboratories at ARI Mlingano in Tanzania and KEPHIS Muguga in Kenya. One of its expected products was a manual for the propagation of African agaves.

Although the project was developed in Tanzania and Kenya, the results of the project will be made available to other countries where sisal and henequen are grown.

The methodologies implemented in these laboratories are based on the ones developed for the micropropagation of Mexican agaves (henequen and tequila), which have also been successfully applied to other species of the genus *Agave* sp.

Because of the intended widespread use of the methodology, applicable to all the species of the group, it was decided to design the manual as a general guide for the propagation of agaves.

It presents the methodologies and their use in the context of agaves' specific problems and their possible solutions and, in this sense, it is very much oriented to the practical use of tissue culture technology to solve problems. Although it is primarily directed to laboratories that might implement the techniques, it was also written with a wider public in mind, with the hope that growers, producers and decision makers might find some useful information on the subject.

Manuel L. Robert
International consultant and
Research scientist

Executive Summary

Executive Summary

A basic method for the rapid micropropagation of *Agave* species, based on research carried out at the Centre for Scientific Research of Yucatán, México, is described in full detail. This method, originally defined for henequen (*Agave fourcroydes* Lem.), has been enriched by the experience of culturing other agaves both in México (*Agave tequilana* Weber var azul and *Agave angustifolia* Haw.), and in E. Africa (*Agave sisalana* Perrine, the hybrid H11648 and others).

Focusing on the biological characteristics of the agaves (Chapter 4), the methods are described step by step, from the selection of the right parental materials to the establishment of the new propagated plants in nurseries or plantations.

Although the method has been successfully used to propagate several other *Agave* species and it could be used for many others, it must be remembered that micropropagation deals with living plants and tissues that vary from each other in their genetic characteristics (morphological and physiological) and under the influence of the environmental conditions that surround them. In consequence, some species might develop better under slightly different conditions. Chapter 5 focuses on the main cultural conditions that should be revised, if problems arise, with suggestions about which experimental variables to consider.

Transplanting *in vitro* plants to soil is a critical transition in the micropropagation process because these plants present morphological and physiological abnormalities that make it difficult for them to survive *ex vitro.* The plants require special care to help them slowly adapt back to normal conditions. The consecutive steps required are discussed in detail in Chapter 6.

Microbial contamination can be one of the most difficult technical problems to solve and can be the source of enormous losses in micropropagation. A great deal of effort must be made to eliminate the microorganisms that accompany the plant tissues from the field and, equally importantly, to prevent the entry of new ones after the cultures have been initiated.

Little is known about the identity of the microorganisms that contaminate the *in vitro* culture of agaves and it is important to create awareness on the need to generate this information (Chapter 7). Though the techniques for this are beyond the scope of most tissue culture laboratories, a general knowledge about them and where they can be implemented is important. All tissue culture laboratories should be able to implement at least the basic techniques for the detection and isolation of microorganisms in order to be able to take preventive actions and to make sure that the plants that are being propagated are not contributing to the dissemination of pathogens.

Micropropagation is a technique to produce large quantities of plants and to solve specific production problems. Large scale propagation, however, requires a very different production system from that used in research laboratories. It is essential for it to be organized as a commercial enterprise where efficiency and efficacy determine the basis of the strategy to be followed. This situation is analyzed in Chapter 8 and some purely theoretical examples are given as to how such a strategy could be implemented. Every variety must be handled differently according to its own characteristics, the amounts of plants to be produced and depending on the delivery calendar. Large scale propagation should be technically supervised by a competent scientist but administered by a trained administrator.

The method presented here is not the only method that has been reported to culture agaves *in vitro* and therefore, to give a whole perspective of the field, a revision of the literature is included (Chapter 3).

At the time of writing, the method that is described here is, in our opinion, the most suitable currently available for rapid and efficient large scale micropropagation of agaves. However, it should also be stressed that new methods are being developed on the culture of somatic embryos and the use of bioreactors (mentioned in Chapter 9) and that a great deal of research in this field is being carried out in various parts of the world. Consequently, it is to be expected that on-going scientific research will produce improvements and better techniques in the near future.

Chapter 1

Introduction

1.1 The uses of agaves

1.2 The need for research and innovation

1.3 The role of plant biotechnology

Chapter 1

Introduction

1.1 The uses of agaves

The genus *Agave* is composed by more than 250 species that are mainly native from Mexico and Central America covering an area that extends to the southern United States and the northern part of South America. Its name comes from the Greek word "noble", referring to the long flowering stalk, characteristic of this plant. Agaves are adapted to a wide variety of habitats from forests and steep slopes to plains, deserts and coastal areas. They can survive in temperatures that range from -9° C to 41° C and altitudes which extend from sea level to 2,400 m.

Evidence suggests that agaves have been important for human activities for thousands of years, mainly as a source of drink and fibre. Hard fibre, extracted from their leaves, was the basis of Yucatan's (in southeast Mexico) wealth at the beginning of the 20th century and it still contributes to a number of economies, notably those of Tanzania, Kenya, Brazil and Cuba, whose fiber is converted into rope, sacks, carpets and crafts etc. Agaves also have a long history in Mexico of being transformed into alcoholic beverages such as tequila, mezcal and pulque. With over 580 brands, tequila has enjoyed the fastest global growth of any spirit in the past few years and the value of the industry's annual exports, mostly to the USA, currently stands at some 200 million dollars.

Agaves potentially most valuable trait, however, in an age of increasing water scarcity, may prove to be their excellent adaptation to severe water limitations. Their photosynthetic system (CAM) fixes CO_2 during the night and stores it until the day light converts it into sugars. This allows them to keep their stomata closed during the day, thus avoiding loss of water through evapo-transpiration.

Agaves possess a thick layer of epicuticular wax which prevents desiccation of tissues and their large root system makes them very efficient at capturing whatever small amounts of water are available in the vicinity. In a world where water is rapidly becoming the number one commodity for human survival, the relevance of useful species that can survive in harsh conditions can hardly be overestimated.

Their long root system also plays an important ecological role preventing soil erosion in arid and semi-arid areas where the plants are commonly used as fences and wind shields.

Other uses include the pharmaceutical and food stuffs industries: new medical uses are being developed for agave derived steroids and some have found an important niche in the elaboration of products for the control of ammonia in the excreta of animals. More recently, agaves have started to be considered as an important source of fructanes, particularly inulin, for the food industry. The use of agave cellulose for paper making (specialty papers) continues to be a viable possibility, as shown by the economic support the CFC/UNIDO project in East Africa has received over the past seven years. Finally, there is an increasing demand for the more attractive agaves such as *Agave victoria reginae* and some variegated varieties of *Agave angustifolia* Haw. (marginata) as ornamental species.

1.2 The need for research and innovation

Agaves have been thoroughly studied from a taxonomical, ecological and physiological point of view. However, in spite of their economic importance, they have been seriously neglected by research directed towards practical applications. This fact was painfully brought home to the tequila industry when the agave plantations were hit by a rampant fungal disease, destroying 20% of the total agave crop, which caused a critical shortage of plants and dramatically increased their price.

In order to generate the knowledge that will lead to technological innovation and a more efficient commercial use of agaves, it is first necessary to investigate a number of problems that many of them share:

- Genetic homogeneity: There is little knowledge of the degree of existing variability in the natural and cultivated populations of agaves which, in spite of their economic importance, have almost never been genetically improved, largely because of their asexual propagation and long life cycle. It is probable that many of the disease related problems that affect some agaves are due to limited genetic variability, something to be expected from the plants´ reproductive characteristics and the dominant agricultural practices.
- Pests and disease: This is an almost unstudied topic which should be given serious consideration if agaves are to be successfully cultivated in the era of open markets and global trade, where pests and disease can be dispersed at unprecedented speed. Preventive strategies to safeguard against the potentially disastrous

consequences of the unintentional spread of pests and disease are urgently needed.

- Over exploitation: Some industrial products are extracted on a massive scale from wild agave populations, putting their survival at risk; this is the case of bacanora and yuca in the north of Mexico.
- Diversification and commercialization: The majority of agave-derived products (with the exception of tequila) present a number of problems, foremost amongst which are: inefficient processes, lack of quality control, low prices and unstable markets. At the same time, other products and sub-products exist which, in spite of their potential, so far remain commercially unexploited. Such is the case of cellulose wastes, fructose derivatives and steroids for new medical uses.

A decade ago it was thought many agaves were on the verge of disappearing because their products and/or uses were rapidly being abandoned. The rise and fall of different agave products over the last decades, however, shows that present trends are not necessarily indicative of future demand. What is clear is that the decisions taken today could play a fundamental role in transforming agaves' difficult present into a more promising future, with new opportunities. Whether and to what degree this promise is fulfilled will depend, to a large degree, on how much investment is made now to increase our knowledge of these plants, in order to develop more efficient and sustainable ways of using them. Only by heavily investing in science it will be possible to meet the high standards of quality and eco-efficiency required by global markets in the 21st century.

1.3 The role of Plant Biotechnology

Plant biotechnology and, in particular, tissue culture offers the right tools to tackle some of the above problems. Tissue culture can play a central role in the generation of new genetic variability and the development of basic tools for long term genetic conservation.

At present, micropropagation techniques are available for the rapid production of new varieties and clones for plantations, as well as for the rescue of endangered varieties. They are also available for the production of healthy, certified lines which should be seriously considered as a means for preventing the dispersal of diseases.

The rest of this document deals with how to implement micropropagation techniques for the benefit of agaves and the industries based on them.

Chapter 2

Genetic Improvement

2.1 Introduction

2.2 The reproductive biology of agaves
- 2.2.1 Sexual reproduction
- 2.2.2 Asexual propagation

2.3 Problems associated with the genetic improvement of agaves

2.4 Genetic improvement of agaves
- 2.4.1 Is there enough genetic variability in the populations of agaves?
- 2.4.2 Selection
- 2.4.3 Production of hybrids through controlled polination
- 2.4.4 Open polination

2.5 Biotechnological alternatives

Chapter 2

Genetic Improvement

2.1 Introduction

The productivity of all agricultural systems is based on two basic aspects: the genetic resources of the crop and the agricultural practices employed.

Of course, the environmental conditions play an important role in the levels of productivity but there is not much one can do to change them. It is possible, however, to adapt to these conditions with the right varieties and the right practices. The continuous selection and genetic improvement of crops is therefore a key strategy to improve productivity. However, in spite of their economic importance, the study of the genetics of agaves is a topic that has been greatly neglected. Very little is known regarding their sexual reproductive biology and the degree of genetic variability that exists in the natural or cultivated populations. It is possible that many of the problems that have occurred in the recent past, such as the bole rot epidemic in the tequila plantations of Jalisco in México, are due to a limited genetic pool which is a consequence of their traditional way of vegetative propagation and to the prevailing old agricultural practices.

With the exception of the programme that produced the famous hybrid H 11648 in Tanzania during the first half of the 20th century (Lock, 1962), there has been no other genetic improvement initiative anywhere in the world. This could be due to the biological characteristics of most agaves, such as their long life cycle and limited sexual reproduction which made it very difficult to study them in the past.

Some agaves are not even cultivated; they are taken from the wild populations whether for the extraction of fibre (lechugilla), alcoholic spirits (bacanora), or steroids (yuca). This is not only an inefficient method of harvesting but one that endangers the biological resources due to overexploitation which is happening in the case of the yucca and bacanora populations in the north of Mexico.

However, this situation is changing due to the introduction of cellular and molecular biotechnologies that can increase the speed of propagation of these plants, analyze the variability at the molecular level and induce the introduction of new genetic variability. Other applications of biotechnology to the cultivation and exploitation of agaves refer to the control of disease dispersal and conservation of the germplasm.

2.2 The Reproductive Biology of Agaves

Agaves can propagate both sexually and vegetatively; however, for reasons that could be due to the characteristics of their habitat and their genetic nature, these plants use asexual propagation as their main means to multiply and preserve the species. Figure 2.1 shows a schematic representation of the reproductive structures of agaves.

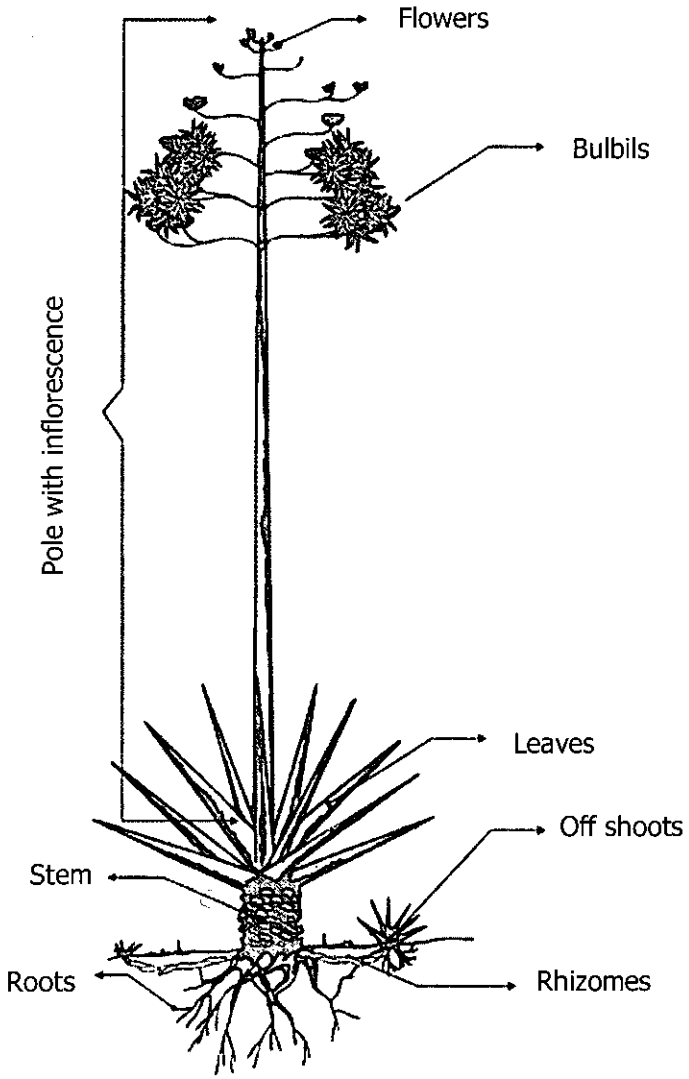

Figure 2.1 General structure of an agave plant showing its sexual and vegetative reproductive structures (modified from Eastmond et al., 2000).

2.2.1 Sexual Reproduction

Towards the end of their lives, the agaves suffer physiological and morphological changes that lead to the formation of a flowering pole. This event is the beginning of the end for the plant, since it occurs as a result of the apical meristem stopping the production of new leaves and being transformed into a flowering structure. The pole grows very rapidly (6 cm/ day) and this is the reason why many growers cut it as soon as it appears in order to prevent the pole from using up all the plants reserves.

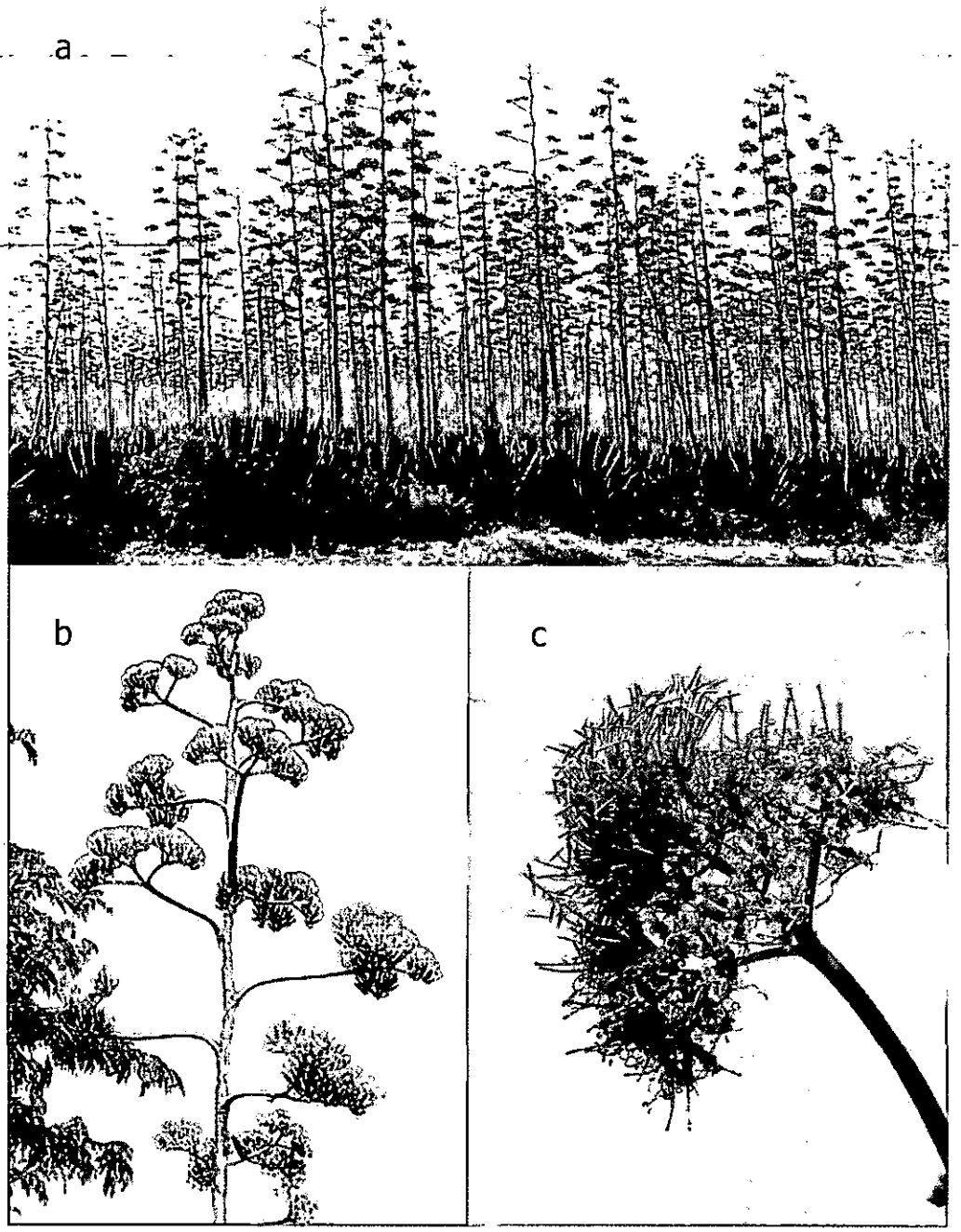

Figure 2.2 a) Poles of H 11648 at Nakuru, Kenya; b) A henequen inflorescence showing the different stages of development of the branches; c) A fully developed branch with mature flowers.

The inflorescences are large, 3 to 5 meters tall in some species (figure 2.2) and covered with numerous bracteal leaves. Flowers are bisexual (hermaphrodite) and form in lateral racemes (Figure 2.2b), starting from the lowest branches. Flowering continues for several weeks from the base upwards, so that after the first few days the inflorescence presents flowers in all stages of development (Piven et al., 2001).

Polination is mediated by insects, birds and bats (Gentry, 1982) and its efficiency is very variable depending on the species. Pollen production can be abundant but its viability is low. Although large numbers of fruits are produced (Figure 2.3a), seed production also depends on the density of the population which determines the amount of pollen available and the proportion of fertilized seeds within them can be very limited (Figure 2.3b).

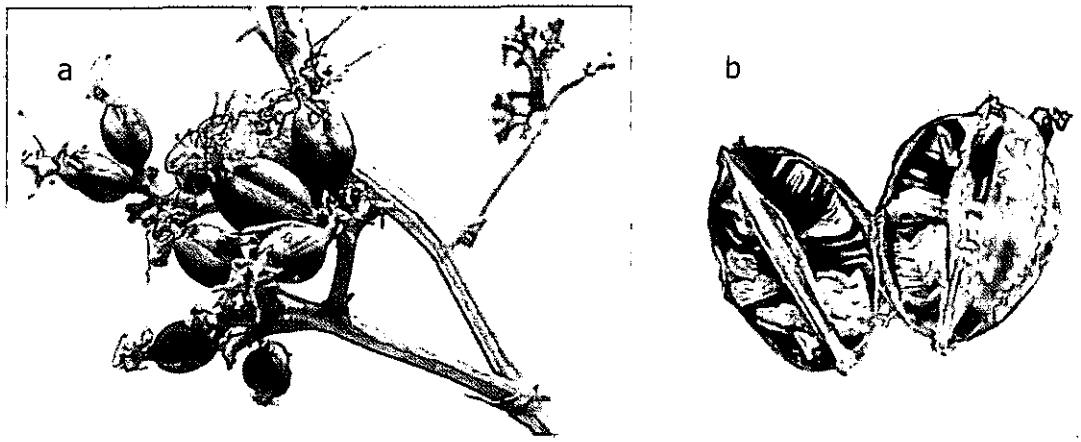

Figure 2.3 a) Mature fruits in the inflorescence, b) Open fruits showing a few viable seeds.

Agaves show a great deal of variation with respect to their levels of ploidy (see chapter 4, Table 4.1) from diploids to octoploids with a basic X number of 30 (Castorena et al., 1991). The polyploids, particularly the pentaploids, (*Agave fourcroydes* Lemaire and *Agave sisalana* Perrine) do not produce normal seeds. *A. sisalana* does not even set fruit and *A. fourcroydes* only seems to produce aneuploid seeds with a very high number of chromosomes (Robert et al., unpublished results).

2.2.2 Asexual Propagation

Although agaves possess sexually reproductive structures, practically all of them propagate asexually by means of rhizome offshoots and bulbils. The reason for this may be found in the arid environmental conditions, in which many of them live, which are not favorable for the germination and establishment of seeds. Asexual reproduction, on the other hand, means that the offsprings are ensured support from the mother plant until they are capable of an independent existence.

The rhizomes are subterraneous shoots that grow away from the mother plant to eventually emerge and form a new plant that originates from the apical meristem of the rhizome (Figure 2.4). Strictly speaking, the rhizomes are branches that remain attached to the mother plant until they eventually separate and form a new individual.

Figure 2.4 Shoot emerging from the tip of a rhizome in the nursery.

Offshoots from rhizomes are the main source of planting material for many cultivated agaves and cuttings are periodically made to collect them. The offshoots, however, are not complete plants since they lack roots and it is necessary for them to be planted in nurseries before they form them and start growing on their own.

A second asexual propagation mechanism is through the production of bulbils that form in the branches of the inflorescences (Figure 2.5a). These are fully formed plants that originate from buds in the axils of the stems of the fruits (Fig 2.5b). They are not sexually produced plants and originate quite independently of the development of flowers and fruits. The bulbils eventually fall down from the inflorescence and some establish themselves on the ground and carry on growing.

Figure 2.5 a) bulbils in the inflorescence; b) Histological cut showing the bulbil developing from the axil of the flower stem.

In theory, all bulbils from a single mother plant form a clone. However, they appear towards the end of the life of the plant and might have accumulated large amounts of somatic mutations that could mean that they differ from each other and do not form a clone. This is presently being investigated in *Agave tequilana* at the Centre for Scientific

Research of Yucatán. Bulbils constitute the main planting material for sisal and H 11648 in East Africa.

2.3 Problems Associated with the Genetic Improvement of Agaves

The traditional method for the genetic improvement of most agricultural crops is based on crosses and back crosses through controlled pollination. The crossing of plants that show interesting new characteristics leads to the production of superior varieties. In the case of agaves, this method is problematic for various reasons: firstly, because of their long life cycle which, depending on the species, can last between 8 and 25 years and because they only flower once towards the end of their life. In some agaves this is further complicated by the fact that growers cut the poles that will produce the inflorescence in order to stop these organs from using up the reserves stored in the plant and thereby delaying its death. This prevents flowering and, therefore, the natural pollination process that generates variability.

To make things worse, some agaves, such as henequen and sisal, are polyploids and have limited sexual reproduction (sisal does not even produce flowers). Pentaploids do not produce much viable seed (Piven et al., 2001) and the few seeds that are produced show abnormal chromosome numbers (Robert et al., unpublished results).

Henequen, as most agaves cultivated for industrial purposes, is vegetatively propagated, producing between 20-30 rhizome offshoots during its life. Although this is sufficient for the establishment of new plantations, it is not enough to establish a programme of masal selection and cloning of outstanding materials which is the only way to improve the productivity of the plantations (Eastmond et al., 2000).

2.4 Genetic Improvement of Agaves

The only programme to genetically improve agaves was implemented by the British in Tanzania during the first half of the 20th century. The selection and crossing of several diploid agaves produced the only high yielding hybrid that has been cultivated for industrial purposes: the hybrid H 11648 that is still the base of the hard fibre plantations in East Africa (Lock, 1962). The programme was cancelled for political reasons but a few years ago the first steps were taken to build laboratories with a biotechnological capability to support a new initiative in Kenya and Tanzania to boost the development of the sisal industry in East Africa (CFC/UNIDO Project: Product and Market Development of Sisal and Henequen Products).

In Mexico, though research on agaves has been an ongoing activity in several research laboratories, it is only recently that industries have started to take an interest and participate in the development and use of the new technologies. The two largest tequila industries, Tequila Herradura and Tequila Sauza have micropropagation laboratories and are planting millions of micropropagated plants (Del Real Laborde, 2004).

The genetic improvement of agaves will require several different strategies that go from the selection and characterization of existing variability to the generation of new variants through biotechnological methods.

2.4.1 Is there Enough Genetic Variability in the Populations of Agaves?

Genetic variability is the raw material for genetic improvement and it is limited in species that are vegetatively propagated. In theory, vegetative or asexual propagation does not generate new genetic variation and produces genetically identical individuals (clones). How then are agaves so well adapted to extreme conditions and hostile environments? How frequently do mutations appear and how can these be transmitted to the next generations through vegetative propagation?

There is some preliminary evidence coming out from the molecular analysis of the genomes that, though limited when compared with sexually propagated species, there is a certain degree of genetic variability in the wild and cultivated populations of agaves (Gil, Vega. et al., 2001; Reyes, Ph. D., CICY) many on-going studies are expected to further clarify this situation.

This variability can be the basis of a first step to improve the productivity of the plantations. In fact, results obtained in the field with selected ("elite") micropropagated lines of henequen (*Agave fourcroydes*) confirm that this is a real possibility. Selected micropropagated lines developed faster and produced more and larger leaves that nearly almost doubled the productivity (Eastmond et al., 2000).

2.4.2 Selection

Continuous selection is an absolute necessity in any genetic improvement process. Selection of only the best, healthiest and most vigorous individuals (masal selection) for use as planting material is a common practice in many systems and one that should always be applied to agaves (in practice, this advice is not always followed). Although an effective means for maintaining the levels of productivity and controlling disease dispersal, this type of mass selection is not a true genetic improvement process because it does not select the progeny of "superior", mature individuals that have shown their full potential and it does not increase their frequency through clonal propagation. For this

purpose, mass propagation of the best individuals is required and it is here that micropropagation plays a key role in the genetic improvement of agaves, producing thousands of individuals from a single selected mother plant (elite clone) in a short period of time. The selected mother plant can be an outstanding individual from a mature plantation or mutant, a somaclonal variant and, in the future, genetically transformed plants.

Figure 2.6 shows how the selection and cloning of elite individuals displaces the distribution of the population increasing the numbers of plants with the desired trait.

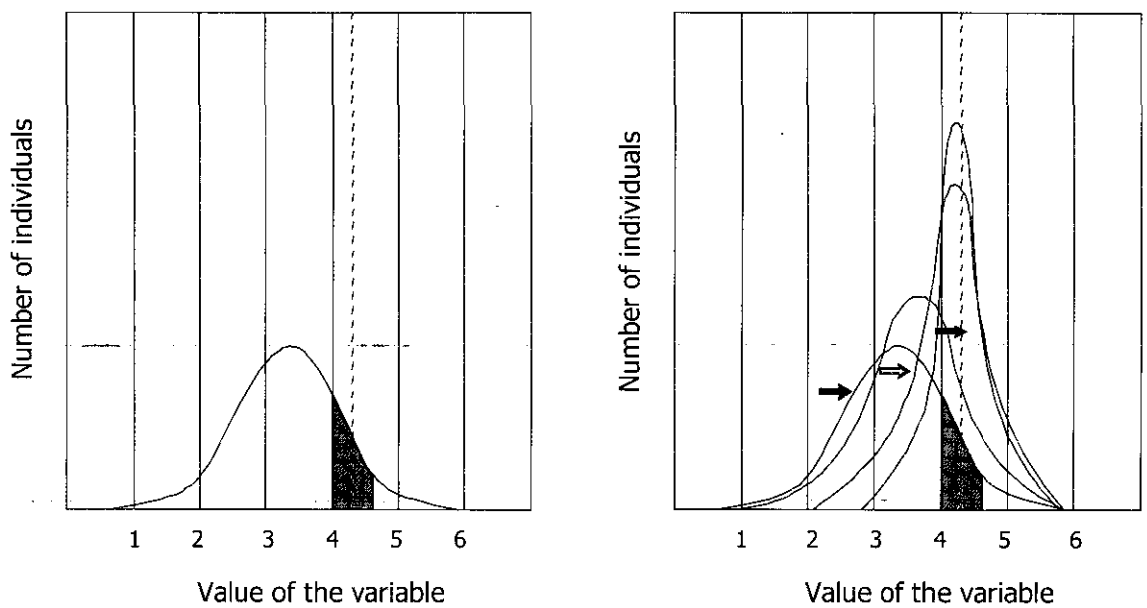

Figure 2.6 A scheme showing how the selection and propagation of an outstanding trait changes the distribution of the population for that character(From Eastmond et al 2000).

2.4.3 Production of Hybrids Through Controlled Pollination

Another strategy for genetic improvement is the crossing of individuals from different species that produces hybrids. This is done through controlled (manual or open) pollination of individuals with new or outstanding characteristics and well established cultivated lines or varieties.

This was the strategy followed by Lock and his collaborators (Lock 1962), who combined the characteristics of two species: the high number of leaves of *Agave angustifolia* Haw with the large size of the leaves of *Agave amaniensis* Trel & Nowell, to generate the family of hybrids that gave rise to H 11648 (*A. angustifolia x A. amaniensis,* back crossed to *A. angustifolia*) the only commercial hybrid widely used in the commercial plantations in East Africa.

Although, this must have been a lengthy process, due to the long life cycle of the species involved and the difficulties to perform it (Figure 2.7), it is still a viable strategy that should be applied to all diploid species of commercial value. For the polyploid species, such as henequen (*Agave fourcroydes*) and sisal (*A. sisalana*), this method is not an option.

Figure 2.7 Selecting flowers for manual pollination.

2.4.4 Open Pollination

Seed produced by open polination could be used to generate a broader variability base for the commercial diploid agaves such as *Agave tequilana*, even if it is only done to increase the variability within the populations.

In order to implement such a programme it is necessary to conserve the flowering poles and collect the seed to establish nurseries from which elite individuals could be selected and cloned. The costs of doing this could be reduced by combining it with normal management practices.

2.5 Biotechnological Alternatives

From what has already been said it is clear that the generation of genetic variability is a key strategy for the genetic improvement of agaves; it is also evident that biotechnology offers the most viable techniques for achieving this aim. Some of these techniques are beyond of the scope of this work so they will only be mentioned here:

- Generation and selection of somaclonal variants
- Induced mutations
- Genetically transformed plants

The important thing to note at this point is that all of the above techniques will require tissue culture and micropropagation in order to multiply the plants with improved traits. Micropropagation is, therefore, a central methodology to implement a genetic improvement programme of agaves, independently of the strategy chosen since, not only it makes the propagation of selected elite materials more rapid and efficient but it also produces high quality, healthy and re-invigorated materials.

Chapter 3

Agave Tissue Culture Research

3.1 A brief introduction to micropropagation

3.2 The organogenic and embryogenic pathways
- 3.2.1 Callus formation
- 3.2.2 Direct organogenesis
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- 3.3.1 Micropropagation through direct organogenesis
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Chapter 3

Agave Tissue Culture Research

3.1 A Brief Introduction to Micropropagation

Micropropagation is the use of *in vitro* culture techniques for the rapid and efficient vegetative propagation of plants. It has been widely used to propagate wild and cultivated plants (George, 1993; Dodds, 1991), and, although applicable to practically all species, it particularly benefits those with long life cycles and inefficient reproductive systems or those whose populations have been greatly diminished (Fay, 1992).

These techniques are employed for commercial purposes in hundreds of laboratories world wide to produce plants for agriculture, horticulture and forestry. According to the European Directory for Tissue Culture (O'Riordain, 1999), more than 500 laboratories, only in Europe, micropropagated 179 million vitroplants in 1996.

The applications of micropropagation to agriculture and conservation can be summarized as follows:

- The rapid propagation of new genotypes
    - Selected "elite" materials
    - Selected mutations and somaclonal variants
    - New hybrids
    - New transgenic lines
- Genetic conservation
    - Propagation of endangered species or varieties
    - In vitro long term germplasm storage
- Production of disease-free plants

The main objective of micropropagation is the rapid production of large numbers of plants to generate clonal lines of high quality materials formed by hundreds or thousands of genetically homogeneous individuals that are identical to the mother plant from which they were generated (true to type).

The micropropagation of any species involves five main stages that were defined by Murashige (1974) as:

- Stage 0: Preparation of the mother (explant donor) plants
- Stage 1: Establishment of cultures *in vitro*
- Stage 2: Multiplication
- Stage 3: Rooting
- Stage 4: Transfer to soil

Stage 0 Includes the selection, pre-adaptation and disinfestation of donor plants to ensure that the explants will be adequate for initiation.

During **stage 1** the tissues that were excised from the mother plant (explants) are incubated and start proliferating along one of the following developmental paths:

- Callus formation
- Organ formation (organogenesis)
- Embryo formation (somatic embryogenesis)
- Meristem culture

Which pathway takes place depends mainly on the type of explant and on the levels and balance of auxins and cytokinins. However, many other chemical or environmental factors such as light intensity are also important.

Stage 2 represents the core of the micropropagation process since it is here that the numbers of plants increase through organogenesis with every new cycle of culture.

Stage 3 is also an organogenic process but is distinct since the formation of roots (rhizogenesis) has to be induced by a different balance of growth regulators after all the shoots have been formed. The emergence of the roots inhibits the formation of new shoots.

Stage 4 is concerned with the transplantation of the new plants to soil and their adaptation to survive outside the culture dish.

3.2 The Organogenic and Embryogenic Pathways

3.2.1 Callus Formation

A callus consists of a mass of undifferentiated cells that are neither stem not leaf nor root cells; they divide in a disorganized way but have the capacity, under the right growth regulator balance, to revert to organized growth and to form new functional tissues.

3.2.2 Direct Organogenesis

Direct organogenesis is the formation of new plants directly from the meristematic tissues present in the explants. Under the influence of the growth regulators, the cells start dividing in an organized manner that gives rise to shoots (without roots). Many shoots can be formed from each explant but the real multiplication process starts when the shoots are separated and induced to form a new round of shoots that grow from their base (adventitious shoots).

This process is repeated once and again, increasing the total number of shoots that are formed from one mother plant (clone).

3.2.3 Indirect Organogenesis

Indirect organogenesis is the formation of new shoots, but not directly from the organized tissues extracted from plants but from an intermediate amorphous dedifferentiated tissue called callus.

The formation of plants through calli (plural for callus) represents an alternative for mass production since the calli can grow very rapidly to form millions of cells from which, at least in theory, millions of new plants can be formed.

This method might, however, present serious disadvantages since disorganized growth also tends to mutate more rapidly and produce variants in the new plants which is not desirable in the production of clonal lines.

3.2.4 Somatic Embryogenesis

Somatic embryogenesis is the formation of plant embryos from somatic cells. It differs from normal embryogenesis because it is not the product of the fusion of a male and a female sexual gamete that results after pollination, but the organized development of an undifferentiated cell that gives rise to an embryo. On the other hand, it differs from organogenesis because the final result is not a shoot or a root but a full plant in its early stages of development.

Although somatic embryogenesis occurs in nature in more than 60 families of plants (including the cruciferae, cucurbitaceae, leguminosae, palmaceae etc), its relevance for biotechnology is based on the fact that it can be induced from many somatic cells cultured *in vitro.*

Somatic embryogenesis can also occur directly from the cells of the explants (direct somatic embryogenesis) or via callus (indirect somatic embryogenesis).

3.3 Agave Tissue Culture

3.3.1 Micropropagation Through Direct Organogenesis

The first studies on the *in vitro* culture of *Agave* species were carried out in the early 80s by Madrigal (1981) who induced the development of axillary buds from *Agave fourcroydes* Lemaire using kinetin (4.64 μM) and IAA (1.71 μM).

In 1982, Frydrich reported the formation of adventitious shoots from the base of bulbils of *Agave sisalana* Perrine, obtaining an average of three shoots per each mother plant.

Robert and collaborators (1987) used high cytokinin concentrations (38.2 μM BAP) to induce adventitious shoots from the explants extracted from the stems of rhizomes of *Agave fourcroydes* (Figure 3.1). This increased the number of shoots obtained from a single mother plant to about 130. The continuous

induction of new adventitious shoots from the base of these shoots trebled the number of plants every four weeks, opening the possibility of producing clonal lines of thousands of individuals in only a few months. A similar procedure was used by Power and Backhaus (1989) to propagate *Agave arizonica* Gentry and Weber.

Figure 3.1 Direct organogenesis from apical meristematic tissues of *Agave fourcroydes* Lem (henequen).

The rapid propagation of *Agave cantala* Roxb*; A. fourcroydes* and *A. sisalana* from rhizome tissues cultured in MS medium supplemented with coconut water (10%) was reported by Binh et al. (1990). With 0.40 μM ANA; 0.49 μM IBA and 2.32 μM kinetin they also achieved a multiplication rate of 3 to 4 new shoots every four weeks.

Santa Cruz-Ruvalcaba and collaborators (1999) described a method to propagate *Agave Parrasana* Berger, a species of ornamental value from Coahuila, México from stem explants cultured in MS media supplemented with 13.3 μM BA.

3.3.2 Micropropagation Through Indirect Organogenesis

Madrigal (1981) also reported the regeneration of henequen plants from calli derived from stem tissues using MS medium supplemented with 4.4 μM BA and 14.7 μM IBA.

In 1987, Robert et al. reported the formation of callus tissue from the internodal regions of rhizomes of henequen (*Agave fourcroydes*) cultured in MS media supplemented with 0.113 μM 2,4-D + 3.82 μM BAP. The induction of shoots, however, required the transfer the calli to Schenk and Hildebrandt

(1972) nutrient media (SH), supplemented with 2,4-D (0.113 μM) and BAP (38.2 μM). It was found that the balance of NO_3/ NH_4 is critical for the organogenic process and that the use of 1mM arginine greatly accelerated the formation of new shoots.

Nodal segments have also been reported as explants for the micropropagation of *Agave sisalana* (Binh et al. 1990; Das, 1992).

Powers and collaborators (1989) reported the regeneration of plants from callus originated from basal segments of bulbils from *Agave arizonica*. The calli were induced with 2,4-D (1.35 μM) while the organogenesis required NAA (5.37 μM) and BAP (38.2 μM).

Calli of *Agave wightii* Drum and Pain cultivated in semisolid and liquid media to study the synthesis of steroids in undifferentiated tissues (Sharma et al., 1979), surprisingly gave rise to bulbil-like structures when cultured on MS medium supplemented with 2,4-D (9.04 μM).

In 1997, Nikam and collaborators reported the formation of adventitious shoots from rhizome and stem explants of *Agave sisalana* both directly and indirectly. The organogenic capacity of the calli remained for more than 32 months.

Hazra and collaborators (2002) reported indirect organogenesis in *Agave sisalana*, employing calli derived from immature leaves grown in MS media supplemented with 2,4-D (9.05 μM) and kinetin (4.6 μM). Of the cytokinins assessed, BAP was the most effective at 26.6 μM, yielding 25 shoot buds per gram of calli.

3.3.3 Micropropagation Through Direct Somatic Embryogenesis

An efficient method for the induction of somatic embryos of *Agave victoria reginae* Moore was developed by Rodriguez–Garay and collaborators (1996b). Leaf explants cultured on semisolid MS media supplemented with vitamins and 2,4-D (1.4 μM), developed somatic embryos in 6 weeks. The embryos germinated after 8 weeks on MS at half ionic strength and 4 additional weeks in half strength SH, both without growth regulators. The hyperhydricity (vitrification) of the plants was reduced using cultured dishes with ventilated tops during the last four weeks in SH.

3.3.4 Micropropagation Through Indirect Somatic Embryogenesis

In 2002, Piven and collaborators reported the generation of somatic embryos from *Agave fourcroydes*. The embryogenic calli (Figure 3.2a) were induced in semisolid MS medium supplemented with 2,4-D (1.13 μM) and BAP (3.82 μM) from segments of stems from *in vitro* cultured plantlets. The formation of embryos (Figure 3.2b) was attained in MS medium with high concentrations of BAP (38.2 μM) in the dark. The embryos matured and germinated in MS medium without growth regulators under a 16 h photoperiod.

Recently, Nikam and collaborators (2003) reported a similar strategy from stems of young bulbils of *Agave sisalana.* The histological observations showed that the embryos had originated from different types of cells (epidermal, sub-epidermal etc.)

Martinez-Palacios and collaborators (2003) also reported the production of somatic embryos from callus induced from stems of *in vitro* grown plants cultured on MS medium of *Agave victoria reginae.* In MS medium with 2.26 μM 2,4-D, 50% of the calli formed embryos.

In theory, this should be the most efficient micropropagation system since every one of the millions of undifferentiated callus cells that can be easily produced, has the potential to divide and give rise to an embryo. However, the induction of embryogenic calli is still very poorly understood and difficult to obtain in many species.

The main risk of using this system is the possibility of generating large numbers of off types. Somatic embryos originating from a large population of cells can have a different cell type origin (Nikam et al., 2003) and also because the undifferentiated cells can accumulate a large amount of mutations.

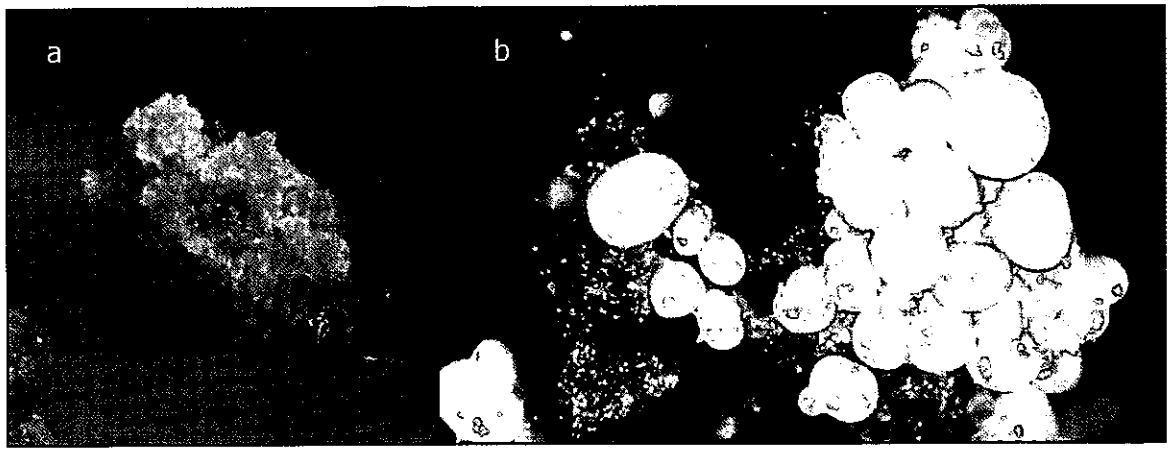


Figure 3.2 a) Undifferentiated calli; b) Indirect somatic embryogenesis in *A. fourcroydes*

With the help of temporary immersion bioreactors (see chapter 10), very efficient systems have been developed for other species such as *Coffea, Hevea* etc. (Etienne & Berthouly, 1999) in which large numbers of embryos can be produce in a relatively short time thereby reducing the risk of mutation. This process however is presently being developed for agaves (see chapter 10).

3.4 Meristem culture

The culture of apical meristems (located at the tip of the plant), or axillary ones (located at the axils of leaves from which new branches will be formed)

constitutes an alternative route for micropropagation. It involves promoting the development of already existing organized regions whose function is, precisely, to form new shoots.

The induction of axillary meristems (by removing the apical one to eliminate the apical dominance) is an effective way to propagate certain crops, while the culture of apical meristems is mainly used to generate disease-free plants (see chapter 8). This is, however, a cumbersome technique since it has to be carried out under the microscope and the final explant is very small (2-3 mm^2) and therefore very susceptible to desiccation and physical damage.

Barragan et al. (2004) reported the successful isolation and culturing of *Agave tequilana* meristems, but, as mentioned before, this is a lengthy and difficult process. However, the objective of producing clonal lines free of microorganisms justifies the effort.

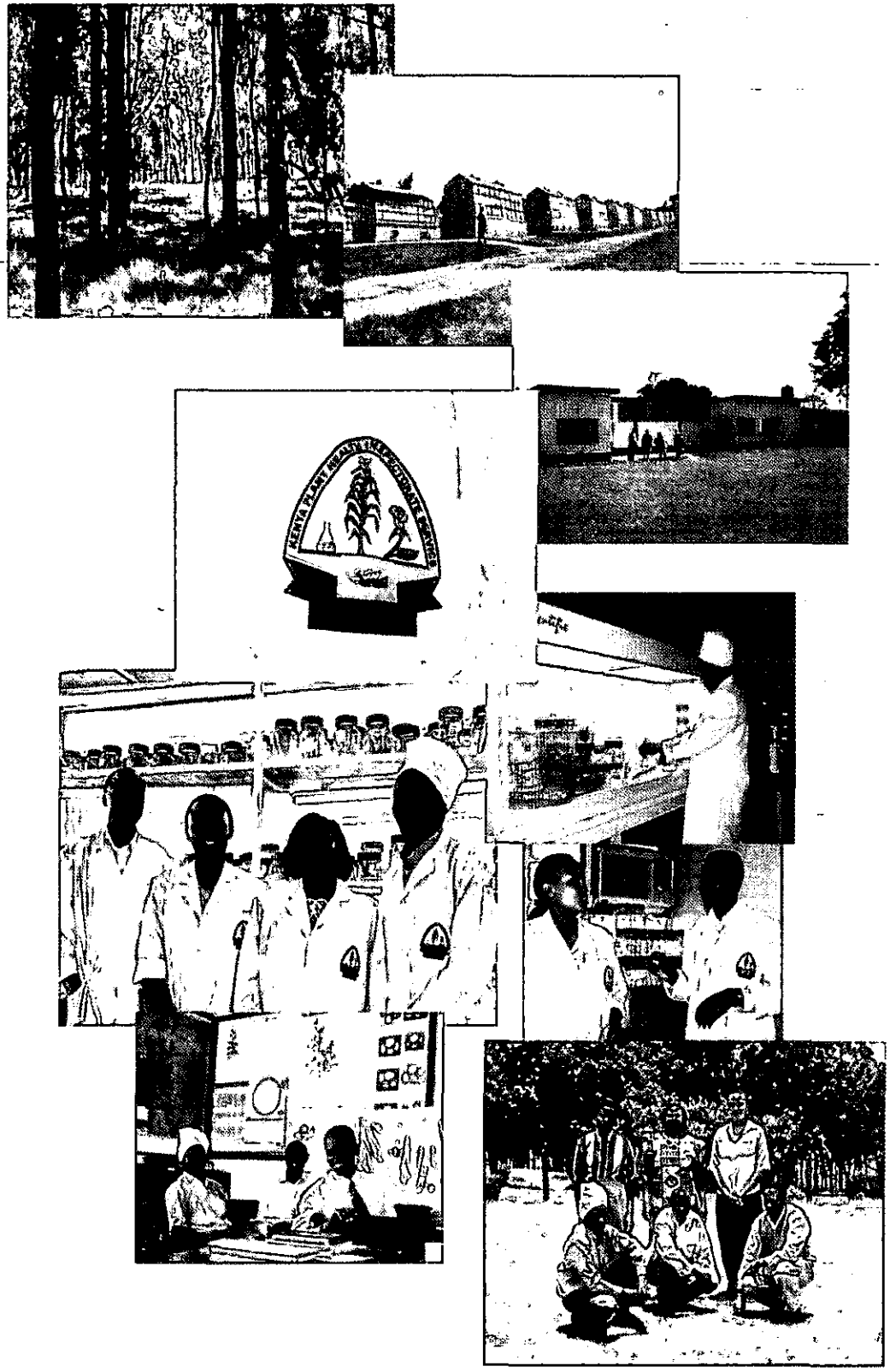
3.5 Present Trends in Agave Biotechnology Research

The first studies of *in vitro* culture of Agave species date from some 25 years ago. After a slow initial development in which only a few groups, mainly in México, worked on this topic, the field has developed to include many species of economic importance, not only in America but in Africa and Asia.

A recent meeting at the Centre for Scientific Research of Yucatán, reveals that the early basic studies (Cruz et al., 1985) have given way to applied research for conservation purposes and genetic improvement of industrial agaves with private industry participating in an important way. The CFC/UNIDO Project (Product and Market Development of Sisal and Henequen Products Project) developed in Kenya and Tanzania over the past seven years included the implementation of tissue culture laboratories for micropropagation of agaves in order to increase the productivity of the sisal plantations and the hard fibre industries. This project is another example of the awareness of the relevance that this technique has for the future commercial development of this important group of species.

Table 3.1 Methodos reported for *in vitro* propagation of several *Agave* species.

Specie	System	References
A. arizonica	Indirect organogenesis from callus	Powers & Backhaus (1989).
A. wightii	Indirect organogenesis from callus	Sharma O.P. & Khanna P. (1979).
A. fourcroydes Lem	Indirect organogenesis from callus	Madrigal-Lugo R. et al., (1981).
A. atrovirens	Indirect organogenesis from callus	Madrigal-Lugo R. et al., (1989).
A. fourcroydes	Indirect organogenesis from callus	Robert ML et al., (1987).
A. sisalana	Indirect organogenesis from callus	Binh LT et al., (1990).
A. sisalana	Indirect organogenesis from callus	Hazra S.K. et al., (2002).
Agave sp.	Indirect organogenesis from callus	Groenewald EG, et al., (1977).
A. tequilana	Indirect organogenesis from callus	Castro-Concha L. et al., (1990).
A. atrovirens	Shoot proliferation directly from the original explant	Villalobos A. V.M. (1993).
A. sisalana	Shoot proliferation directly from the original explant	Frydrych D. (1982).
A. fourcroydes	Shoot proliferation directly from the original explant	Robert ML et al., (1987).
A. sisalana	Shoot proliferation directly from the original explant	Das T. (1992).
A. schidigera	Shoot proliferation directly from the original explant	Rodríguez-Garay B. Et al., (1996b).
A. parrasana Berger	Shoot proliferation directly from the original explant	Santacruz-Ruvalcaba F. et al., (1999).
A. victoria-reginae	Somatic embriogénesis	Rodríguez-Garay B. Et al., (1996b).
A. victoriae-reginae	Somatic embriogénesis	Martínez-Palacios A. (2003).
A. fourcroydes	Somatic embryogenesis	Piven N.M. et al., (2002).
A. sisalana	Somatic embryogenesis	Nikam T.D. et al. (2003).



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Chapter 4

Guidelines for the Establishment of a Micropropagation Protocol for Agaves

4.1 Objectives

4.2 Establishing a protocol

4.3 A method for an efficient micropropagation of *Agave* species
- 4.3.1 Selection of the "elite" mother plants
- 4.3.2 Sources of explants
  - 4.3.2.1 Rhizomes
  - 4.3.2.2 Other sources of explants
- 4.3.3 Pre-conditioning of donor plants
- 4.3.4 Extraction of the meristematic tissues
- 4.3.5 Disinfestations of explants and control of microbial growth
- 4.3.6 Cutting the explants
- 4.3.7 Induction: Culturing the explants
  - 4.3.7.1 Separation of the induced plantlets
- 4.3.8 Multiplication
- 4.3.9 Growth
- 4.3.10 *In vitro* pre-adaptation
- 4.3.11 *In vitro* rooting

4.4 Common Problems
- 4.4.1 Browning
- 4.4.2 Vitrification
- 4.4.3 Seasonal variation
- 4.4.3 Intra and inter specific variation

Chapter 4

Guidelines for the Establishment of a Micropropagation Protocol for Agaves

4.1 Objectives

Micropropagation through tissue culture offers the only means to rapidly generate large numbers of genetically homogeneous clonal lines of elite materials from species with a long life cycle.

Clonal lines of high yielding plants that produce more or larger leaves with a higher fibre content have been used to increase the productivity of the plantations of henequen (*Agave fourcroydes* Lem.) in Yucatan, Mexico. Therefore, the objective of this work is to provide the basic procedure to optimize a protocol for the rapid and efficient propagation of high quality plants from different *Agave* species at minimum cost.

4.2 Establishing a Protocol

What is a protocol?

Tissue culture deals with living plants that vary in their genetic characteristics and physiological conditions, it is therefore of fundamental importance to understand that a protocol is only the best methodology available a certain time to deal with a specific problem. It is neither a universal law nor an unquestionable truth. It is a procedure that has to be adapted to tissues that differ in their genetic and physiological conditions in order to obtain the best possible results from them.

There is no such thing as "the protocol" or "the optimal protocol". The use of very fine chemicals can produce more plants at a higher cost or the use or high concentrations of growth regulators may produce many plants but of bad quality. The best protocol for a specific purpose is the one that produces the largest amount of high quality plants of a specific variety, with the available human and material resources of a particular laboratory, at the lowest possible cost. It therefore needs to be specifically adapted to each laboratory.

The basic protocol recommended was used for the micropropagation of henequen (*Agave fourcroydes*) and has been successfully used to propagate other varieties and species of agaves including *A. fourcroydes* Var. Kitam Ki, *A. fourcroydes* var. Yaax Ki, *Agave tequilana* Weber var azul, *Agave letonae*, *Agave angustifolia* Haw (bacanora), *Agave sisalana* Perrine, as well as species of the genus *Yucca*. Each species produced different results in terms of

multiplication efficiency; rapidity of growth etc. The results varied, not only according to the species in question, but also depending on the genetic properties of the different clonal lines and the seasonal or environmental conditions in which they were grown:

The micropropagation efficiency of *Agave tequilana* varied in a cyclical manner according to the month of the year in which the mother plants were collected (Robert et al., 1992).

Clones of *Yucca valida* generated from seed collected from the same mother plant can vary in their multiplication efficiency from 0 to 5 new shoots produced at every transfer.

Agave tequilana clones grow very differently, depending on the type of culture container employed.

Agave sisalana and hybrid H11648 have been reported to multiply with different efficiencies in Kenya and Tanzania.

4.3 A Method for an Efficient Micropropagation of *Agave* Species

The method described, which permits the efficient cloning of thousands of individuals from a single mother plant, was developed at the Biotechnology Unit of the Centre for Scientific Research of Yucatan (Robert et al., 1987; 1992; 2004) and has been successfully applied to many different agaves (see table 4.1)

The method consists of the induction of shoots from meristematic tissues extracted from "elite" plants, their multiplication through direct organogenesis and the *in vitro* pre-adaptation of the plants which produces a very high survival rate when the plants are transferred to soil.

This method allows the rapid propagation of high quality selected "elite" clones with minimum unwanted genetic variability in the plantations or, conversely, helps to develop and introduce new variability in the form of new hybrids or mutants that would take many years to become available for plantation purposes using traditional methods.

The protocol consists of some or all of the following steps, depending on the variety or the objectives of the work:

- Selection of the "elite" mother plants
- Sources of explants
- Pre-conditioning of donor plants
- Extraction of the meristematic tissues
- Disinfestation of the explants
- Cutting of explants
- Culturing the explants (induction)

- Multiplication
- Growth
- *In vitro* pre-adaptation
- *In vitro* rooting

4.3.1 Selection of Mother Plants

The first step towards the establishment of "elite clonal lines" is the adequate selection of mother plants based on their performance in the field (figure 4.1).

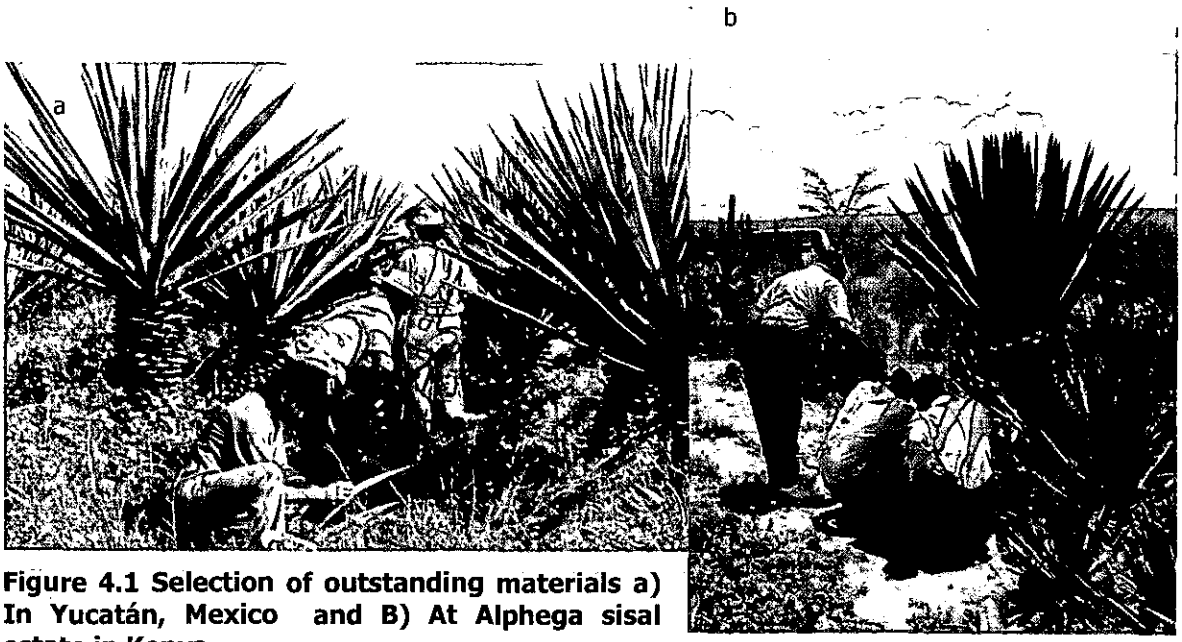

Figure 4.1 Selection of outstanding materials a) In Yucatán, Mexico and B) At Alphega sisal estate in Kenya.

Outstanding healthy individuals are selected in collaboration with growers that know the age of the plantations and the conditions in which the plants have developed. It might be important to know if the plantations have been fertilized or treated with pesticides and how often; if there has been a severe draught or how many times the plants have been cut etc.

The selected plants must be labeled and measured for future reference.

The main parameters for selection are:

- Overall size
- Total number of leaves
- Length and width of the middle leaves
- Thickness and rigidity of the leaves (indicative of fibre content)
- Diameter of the stem
- Presence of marginal spines
- Basal metabolism

Although most of the selection traits are morphological variants (Figure 4.2), in the near future the measurement of physiological characteristics, such as

malate accumulation in the leaves or basic metabolic rates (isothermal calorimetry) in young plants from nurseries or plantations, will speed up the selection and introduction of high yielding lines.

4.3.2 Sources of Explants

Stems of old plants are not a suitable source of explants (meristematic tissue) for the initiation of *Agave* tissue culture. They are hard, fibrous, have a lignified meristem and, most likely, are infected with one or another type of microorganism. On the other hand, selection of elite materials must be carried out on plants that are old enough to show the full range of their advantageous (superior) characteristics.

4.3.2.1 *Rhizomes*

Apical offshoots from rhizomes from selected elite plants are generally a good source of explant (meristematic) tissue and have been successfully used for this purpose in a large number of *Agave* species, generating an average of 25 new plantlets from a single plant.

Vigorous and healthy 25-40 cm. tall shoots (Figure 4.4), preferably still attached to the mother plant through the rhizome, are ideal; they are big enough to yield a minimum of 8 explants and are small enough to be easily transported to the laboratory. It is very important, however, to make sure that the extracted shoot is the offspring of the selected mother plant and not of a neighbouring one.

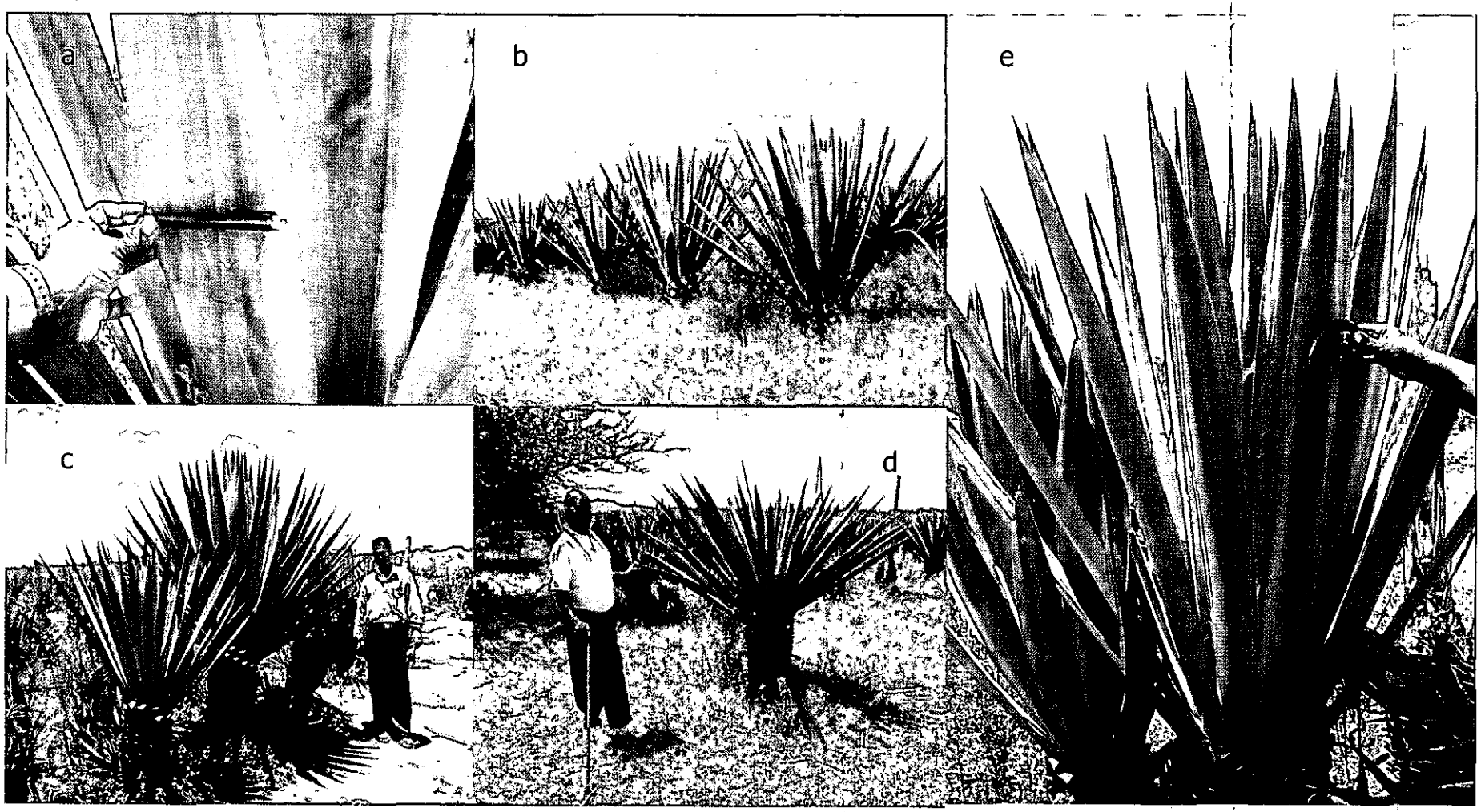

Figure 4.2 Outstanding characteristics to be selected from hard fibre producing agaves: a) Broad leaves; b) *Agave amaniensis*; c) A superb H11648; d) High fibre content (very rigid leaves) and e) Long leaves.

4.3.2.2 Other Sources of Explants

If available, other "younger" biological materials can be used for induction:

- Seedlings from *in vitro* germinated seeds
- Plants from selected bulbils grown in the nursery
- Previously micropropagated plants maintained in the green house or the nursery
- Axillary buds from leaves, rhizomes and inflorescences

In all cases they should preferably be from selected mother plants. The first two options, however, will not represent direct selections and will introduce genetic variability that might or might not be desirable.

The shoots extracted from the rhizomes in the plantations can be used immediately as a source of explants or can be pre-conditioned.

4.3.3 Pre-conditioning of Donor Plants

In some cases the explants from the offshoots from rhizomes do not respond to the induction treatments and turn brown and dry, producing only a few plantlets or none at all.

The reasons for this can be varied:

- Microbial infections, not necessarily obvious or pathogenic, that produce toxins that affect the tissues.
- Environmental stress (very high temperatures or water scarcity) that induces the synthesis of natural inhibitors such as abscisic acid.
- Rhizomes that are "physiologically old tissues" due to chemical influences that diffuse from the old mother plants to the offspring.
- Very small meristematic regions in the tissues used as explants.
- Seasonal variations.
- Extreme susceptibility of the extracted tissues to the chemicals used for disinfection.
- Very low levels of endogenous cytokinins, or very high levels of endogenous auxins that are unsuitable for organogenesis.

The best way to minimize these problems is to separate the rhizomes from their mother plants and remove them from their natural conditions, placing the plants for a few weeks or months in a partially controlled environment in a nursery or, preferably, a green house (Figure 4.3).

Figure 4.3 Pre-adaptation of plants from rhizomes of H11648 in a shaded greenhouse.

Under these conditions the plants will not only be in a homogeneous environment but can be pre-conditioned for tissue culture by removing or counteracting the negative influences that prevent them from generating shoots *in vitro.*

- The plants can be placed individually in bags or pots with sterile soil.
- They can be treated with fungicides or growth regulators to control infections or change the physiological conditions.
- They can be shaded to soften the tissues by reducing the amount of sunlight and temperatures.
- They can be watered at will to eliminate waters stress.

This pre-conditioning has not been extensively investigated because it has not been necessary for most of the *Agave* species that have been successfully cultured *in vitro.*

Although it represents additional work and additional costs to the production system, it must be remembered that it is only for the initiation stage which represents a small percentage of the total micropropagation process and could help to stabilize production all year round.

4.3.4 Extraction of Meristematic Tissues

Before taking the plant tissues into the laboratory, all the leaves are cut (Figure 4.4) and the remaining tissues thoroughly washed with soap and a brush to remove all the soil from them.

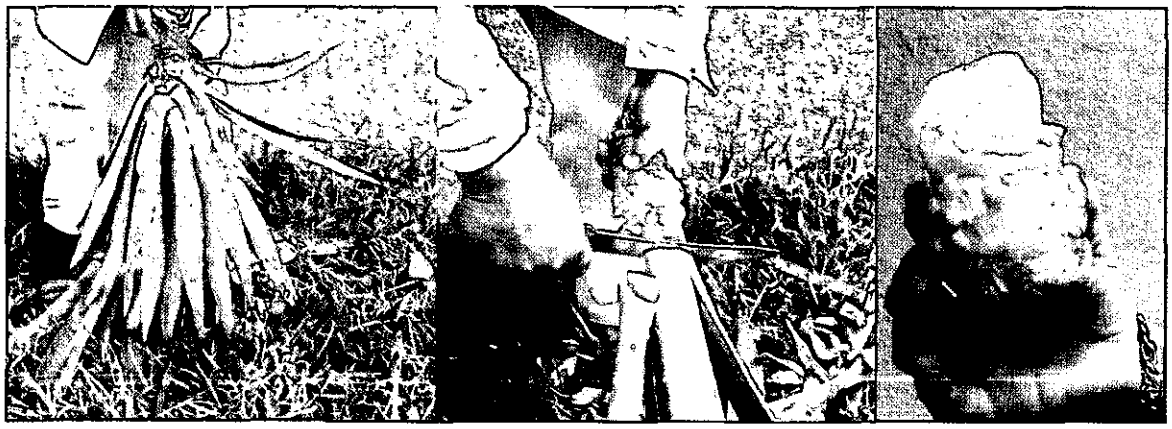

Figure 4.4 Extraction of the meristematic tissues from young selected plants.

As much as possible of the external tissues should be removed with a sharp butchers knife leaving a block of tissue of 6-8 cm per side (Figure 4.5), (note that the size of the explant depends on the species used and the age and size of the mother plant.) The bases of the central leaf primordia at the top of the cube are left in place at this stage to protect the meristematic tissue below them.

Figure 4.5 Cutting the stem tissues before sterilization (a, b and c); d) The block of tissue shows the leaf bases of the primordial leaves below which is the meristem that will be extracted.

The blocks of meristematic tissue extracted from the stems are soaked in extran (2%) or in a tween 20 solution for 30 min (Figure 4.6) before they are taken into an air flow cabinet

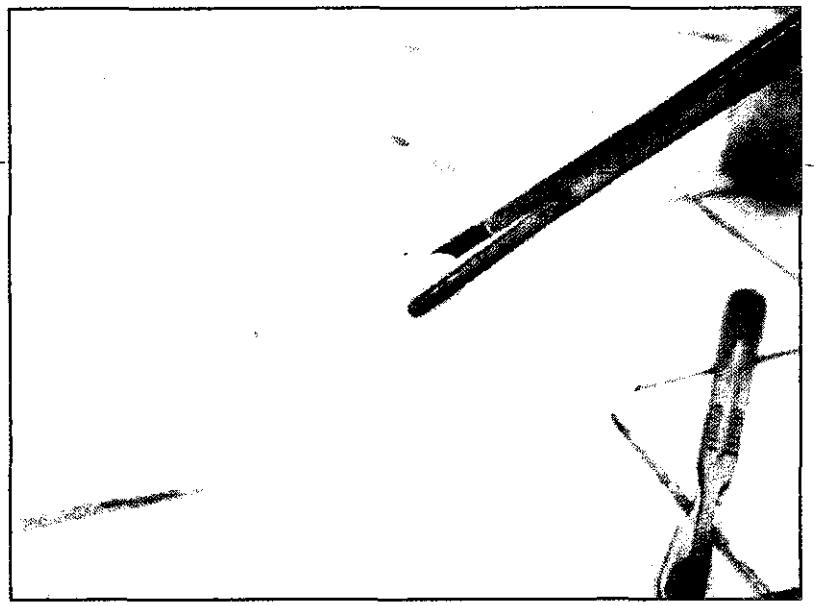

Figure 4.6 First sterilization treatment of the tissues before taking them into the air flow cabinets

4.3.5 Disinfestations of Explants and Control of Microbial Growth

The extracted meristematic tissue is the actual source of the explants that will be cultured *in vitro.*

Figure 4.7 a) Sterilization and b) extraction of the meristematic explant in the air flow cabinets.

The method for the sterilization and cutting of explants is as follows:

- Once in a sterile environment (Figure 4.7), the explants are soaked in 40% commercial bleach (containing 5% active sodium hypochlorite (NaOCl) for 30 minutes, followed by 3 rinses with sterile water.
- The blocks are cut into smaller ($0.8cm^3$.) cubes (Figure 4.8)
- In some cases, where the explants are particularly difficult to clean, the smaller cubes can be immersed in 2% bleach before rinsing thoroughly

with sterile distilled water after which they are placed in the culture media.

- If needed, add 1.6 ml/l Oromex or 0.8 ml/l PPM (Plant Preservative Mixture) to the culture media to control microbial growth in the early stages or induction of lines that are particularly difficult to clean.
- Check the cultures every day and remove the contaminated ones.

4.3.6 Cutting the Explants

The sterile large blocks have the meristematic tissues at their core. To extract these meristems, located right below the base of the leaf primordia, it is necessary to remove the latter completely since their presence will prevent the formation of new plantlets through organogenesis or embryogenesis.

The bases of the leaf primordia can be seen as a pale rosette at the surface of the top of the explant (Figure 4.5d) and can be removed by cutting thin slices of the top surface with the aid of a scalpel until no traces of the rosette can be seen.

The next step is to remove the lateral tissues damaged by the sterilization treatment. Finally, the fibrous tissues at the bases are eliminated in a single cut, leaving a slice of tissue about 1 cm thick.

This meristematic slice can be sterilized again if needed (although it is not recommended) and then cut into smaller 0.8 cm^3 blocks that constitute the explants to be cultured (Figure 4.8).

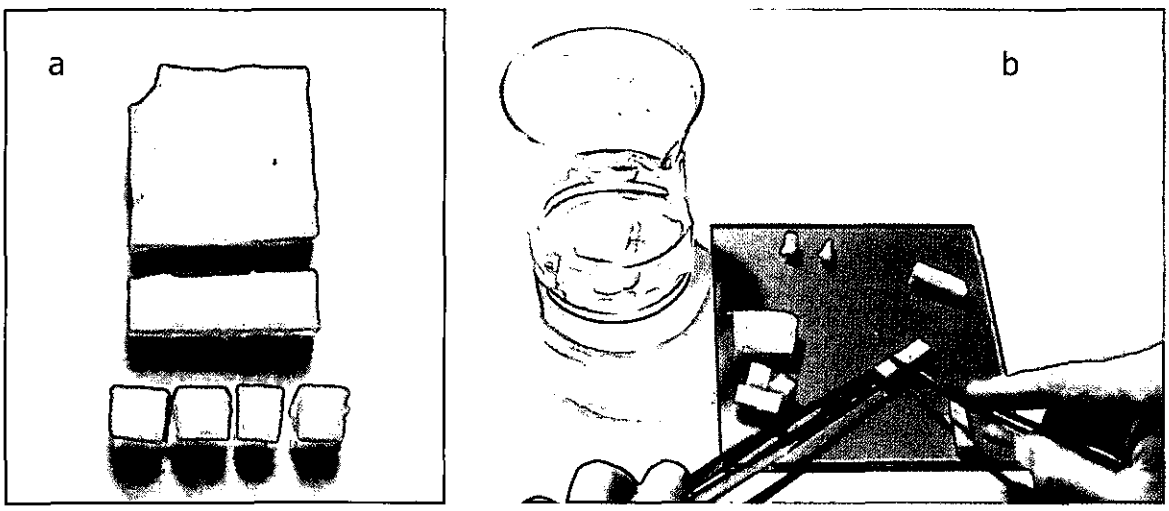

Figure 4.8 Cutting of the explants for induction.

The final size of the slice and the total number of explants extracted from it depends on the size of the base of the mother plant. Small rhizomes can produce as little as 4 explants while larger Kyriazi rhizomes can produce as many as 20.

NOTE: After so much work one is tempted to extract as many explants as possible and many times the second layer of tissue below the first one might be worth culturing.

However, most of the meristematic tissue is in the top block just under the base of the leaf primordia that were removed and this region will produce most of the induced shoots. The decision of how much tissue should be removed comes with the experience and the observation of how different plants respond

4.3.7 Induction: Culturing the Explants

The small (0.8cmm^3) cubes are incubated in baby food jars (Figure 4.9) containing MS-B (MS with reduced nitrogen) media supplemented with 2,4-D (0.025mg/l) and BAP (10mg/l) and solidified with 0.8% agar.

Figure 4.9 Explants of meristematic tissue incubated in induction media.

The jars are incubated in a growth room (Figure 4.10) at 27± 2°C under a 16h. photoperiod (45 µmole/m ^{2} /sec) for 8 to 12 weeks until new shoots are formed on the surface of the explants.

The first shoots start appearing after five weeks and after twelve weeks some 3 to 12 new complete adventitious shoots with a minimum of two leaves, varying in size from 0.5 to 2.0 cm will have been formed on each explant (Figure 4.11).

Some explants do not produce any new shoots; this could be due to the way they are cut. If explants develop only one large central shoot, this is due to the inadequate removal of the pre-formed leaf primordia that just continues growing. What is needed for induction is the undifferentiated meristematic tissue that lies below.

Figure 4.12 illustrates what happens if the meristem region is too small, or the block is cut badly leaving the meristem to one side: only the explants with

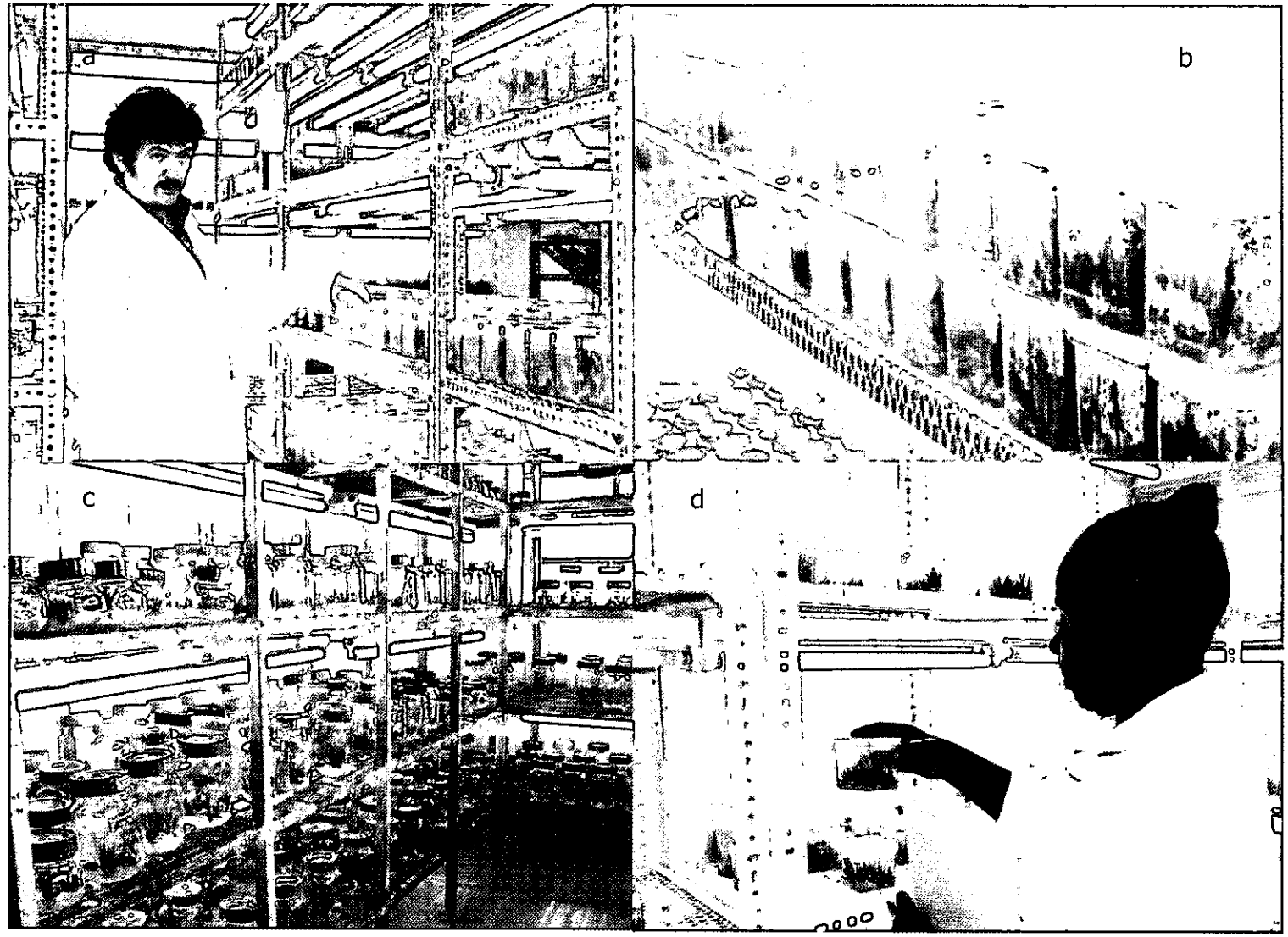

Figure 4.10 Growth rooms for agave tissue culture at: a) and b) CICY, México; c) KEPHIS, Kenya and d) ARI Mlingano, Tanzania

meristematic tissue will give off new shoots. This is not due to dormancy but to the lack of viable meristematic tissue in some of the explants.

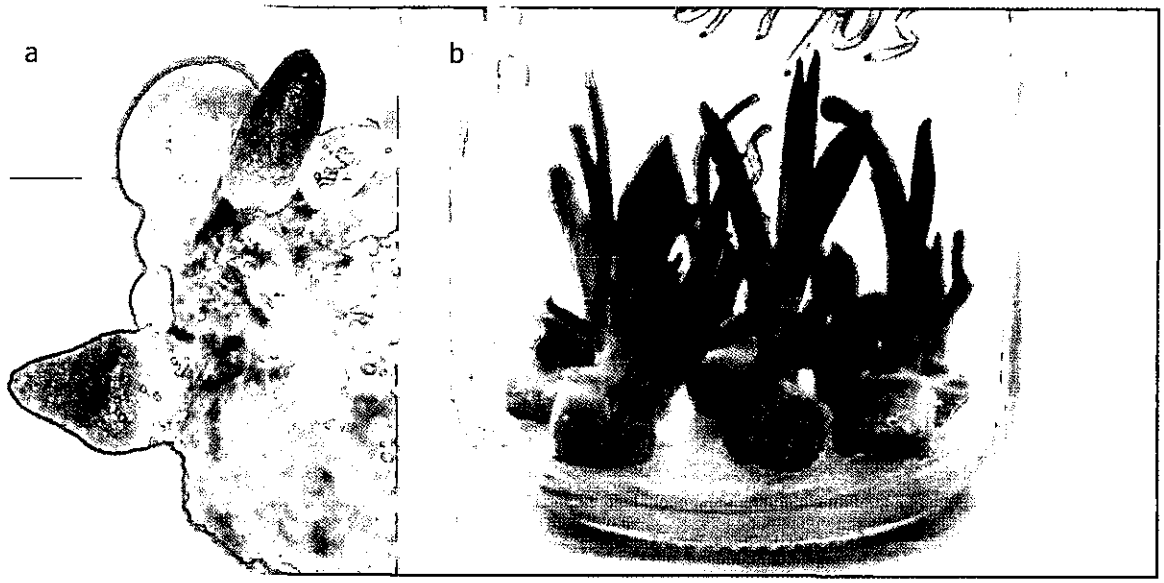

Figure 4.11 Induction of shoots from the meristematic tissues.

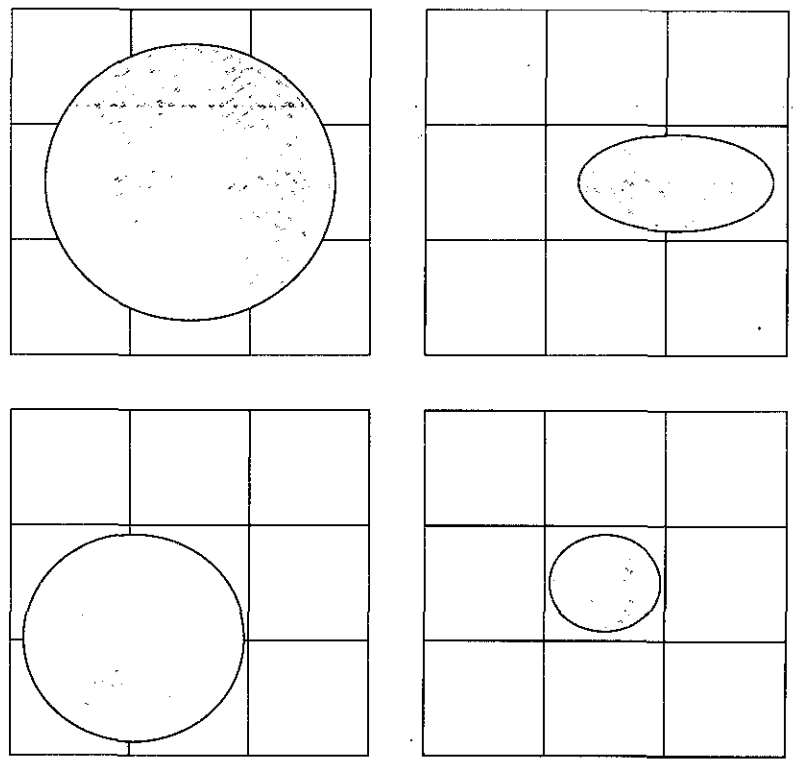

Figure 4.12 Diagrammatic representation of the cutting of the explants into 9 blocks and hypothetical sizes and positions of the meristematic tissue (shaded circles). The blocks without meristematic tissue will not produce shoots.

The size of the Kyriazi variety explants offers a unique opportunity to analyze and position of the meristematic tissue. Maintaining the position and orientation of the explants during induction reveals that the central part of the top level contains most of the meristematic tissue and produces many shoots, while the others will never be induced (Figure 4.13).

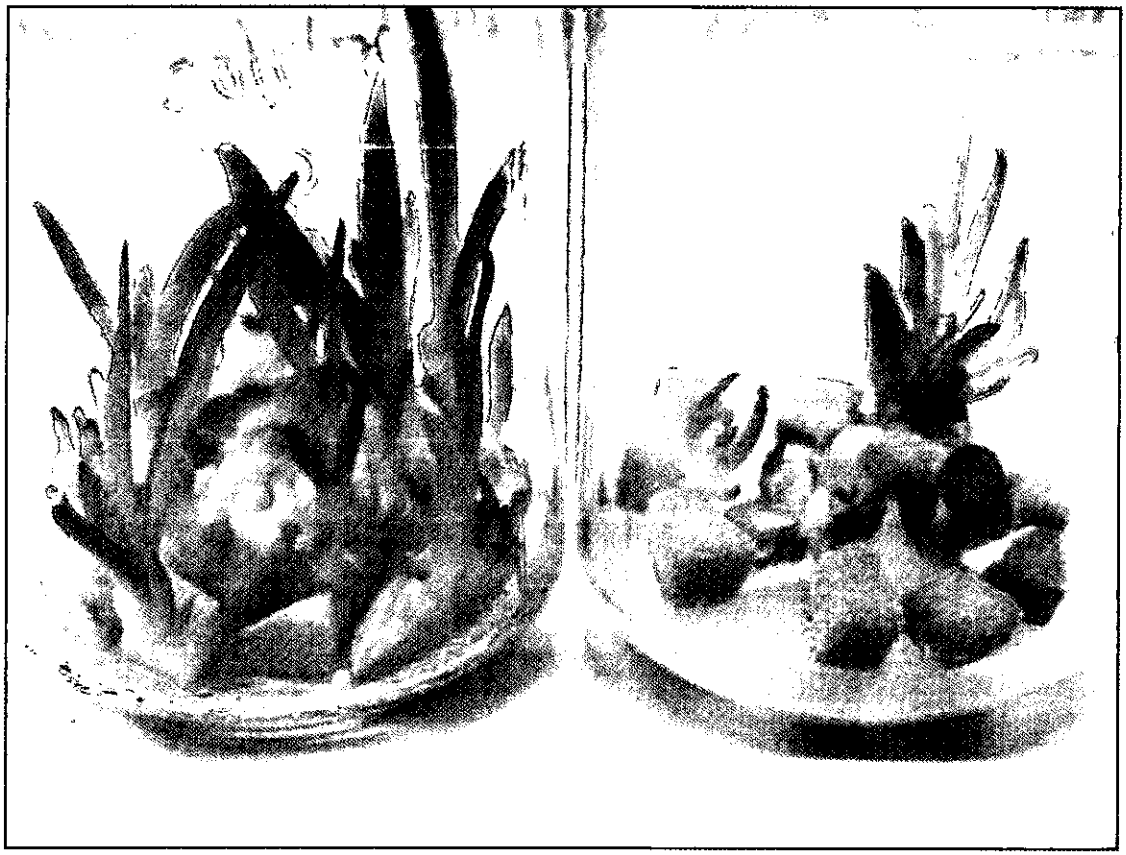

Figure 4.13 Shoot induction in the top and lower levels of the explant cuttings. Note that in the lower level less explants produced less shoots.

4.3.7.1 *Separation of the Induced Plantlets*

In some cases, the new shoots can be separated by gentle pushing with the back of the scalpel or by cutting them apart with the blade. The individualized shoots or groups of three of them are then sorted by size and transferred to magenta boxes to grow and multiply.

It is interesting to mention that the new shoots seem to be formed through two different developmental pathways: organogenesis and somatic embryogenesis (Barredo et al., 2004).

4.3.8 Multiplication

This stage represents the core of the micropropagation procedure since it is here that the number of plants will be increased to the amounts needed (Figure 4.14).

The plantlets are placed in magenta boxes containing 50 ml of MS-B medium, supplemented with 6BAP (1.0 mg/l) and 2,4-D (0.025 mg/l) solidified with 0.175% agar and 0.175% gel rite, and the boxes are incubated in a growth room at 27 ± 2°C under a 16h photoperiod (70µmole/m^2/sec) for 4 weeks.

New adventitious shoots form at the base of the shoots (Figures 4.15) but also multiple new shoots form from axillary buds. The average numbers of new shoots can vary widely from species to species but also from one clone to another within the same species.

The multiplication factor (average number of new plants produced in each transfer) determines how many transfers will be required and how long it will take to produce a certain number of plants.

The new shoots are very variable in size (Figures 4.16) and it is recommended that they are graded and transferred separately in order to maintain as much homogeneity as possible in the culture dishes:

- Small: 0.5-1.0 cm.
- Medium: 1.0-2.0 cm.
- Large: larger than 2.0 cm.

The first two can be transferred to multiplication medium to continue increasing the micropropagated biomass or can be transferred to growth medium (see below) to allow them to reach the right size for pre-adaptation.

The large ones can be transferred directly to growth and pre-adaptation.

The cycle can be repeated as many times as necessary to produce the required number of plants for each cultured line.

4.3.9 Growth

Micro propagated shoots are too small to be taken out of *in vitro* culture and they must be given the opportunity to develop further by keeping them in the same culture medium without growth regulators at 27°C and a 16 h photoperiod (70µmole/m2 /sec) This will allow them to grow, using up any growth regulators that might have accumulated during the multiplication transfers.

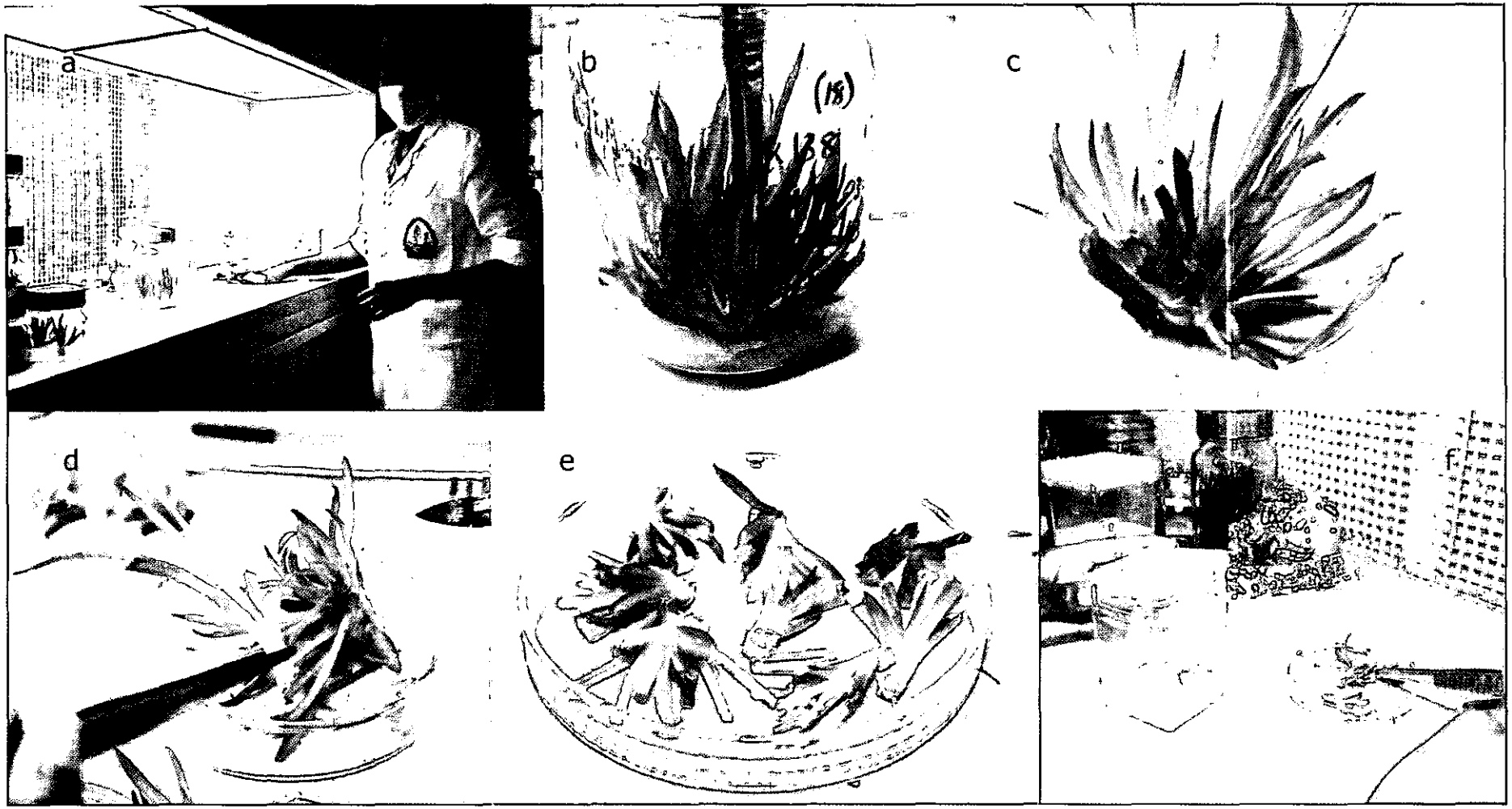

Figure 4.14 Multiplication of K 138 at Kephis in Kenya: a) Preparation of the sterile areas b) and c) Induced shoots; d) and e) Separation of groups or individual shoots; f) Transplanting to multiplication media for a new round of propagation.

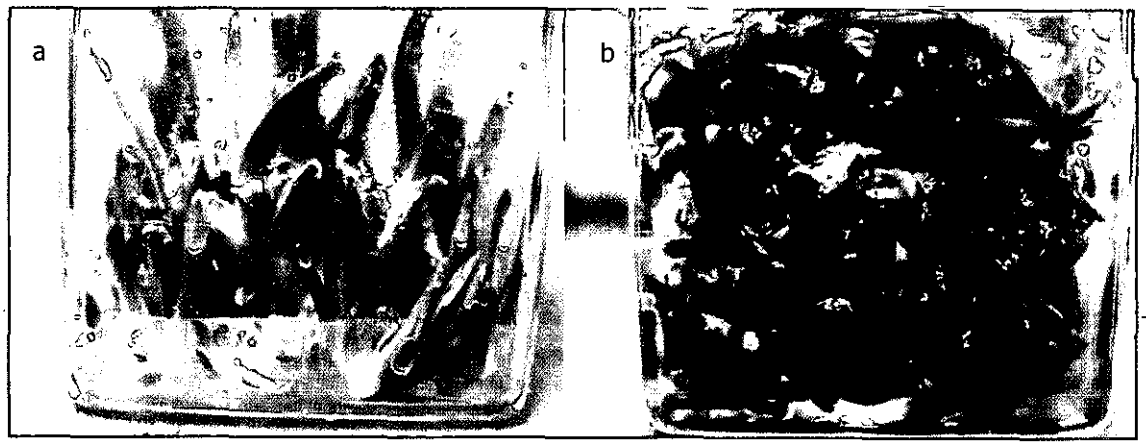

Figure 4.15 Adventitious shoot production during the multiplication stage.

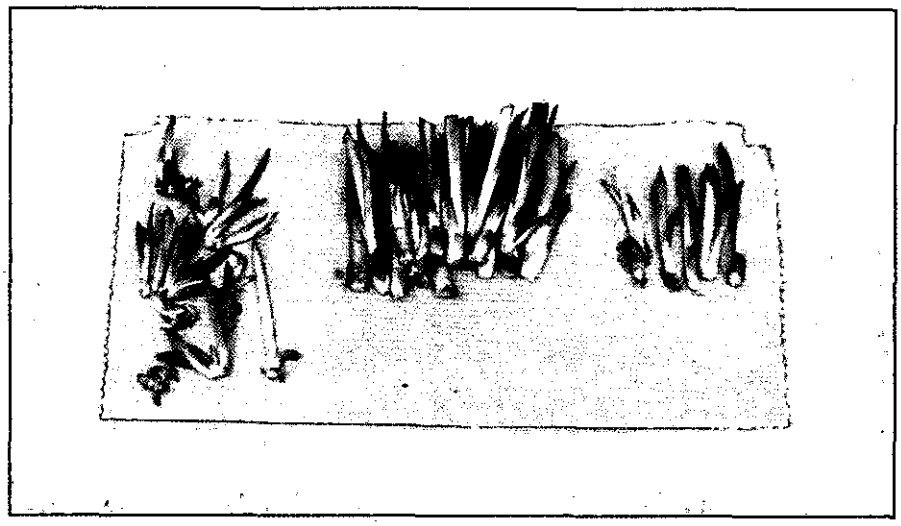

Figure 4.16 Sorting of micropropagated shoots before a new transfer.

4.3.10 *In vitro* Pre-adaptation

The leaves of the vitroplants are not normal leaves. They have abnormal stomata that, in most cases, are not functional and lack epicuticular waxes which make them extremely susceptible to desiccation through rapid loss of water. Furthermore, due to the high concentration of sugars present in the culture media, the plants do not photosynthesize efficiently and suffer when they are removed from the protective and aseptic *in vitro* environment and are planted on soil.

It is therefore recommended that the last growing stage takes place in pre-adaptation media without sugars and growth regulators and with higher gelling concentrations that will limit the availability of water. It is important that these plants are placed on the shelves with the highest possible light intensity (110µmole/m^2/sec) or, preferably, under natural light.

These conditions will help the new plants to start preparing for the shock of the dryer and less nutritious *ex vitro* environment.

Figure 4.17 Pre-adaptation. Note the darker and glaucous color of the leaves due to initial deposition of epicuticular waxes

4.3.11 *In vitro* Rooting

The plants can produce roots in the pre adaptation medium (Figure 4.18) but a small amount of auxin can be added taking care that no callus is formed. However, it is not essential for their survival to root plants *in vitro*; they can be transplanted without roots, providing that adequate care is taken to protect them from desiccation once they are on soil.

Figure 4.18 In vitro root formation.

Chapter 6 deals with the handling of the plants from the moment they leave the *in vitro* container until they are sown in the plantations.

4.4 Common Problems

There are several problems that are recurrent in tissue culture and must be continuously checked and recorded:

4.4.1 Browning

The harsh handling of tissues during extraction and cutting, or the effect of high concentrations of chemicals during disinfestations or culture will produce the accumulation of phenolic compounds in the explants. These phenolics can induce oxidation which causes the tissues to turn brown, arresting their development. A milder disinfestation treatment might reduce browning.

The use of fibrous tissues from old donor plants is another possible cause of browning

4.4.2 Vitrification

The high concentrations of growth regulators used or the water potential of the culture medium (Castro Concha et al., 1990) can induce abnormal hyperhydricity that produces the vitreous appearance of the tissues known as vitrification (Figure 4.19). This problem can be controlled by means of a more concentrated gelling or using ventilated tops in the culture dishes. Santamaria et al. 1995).

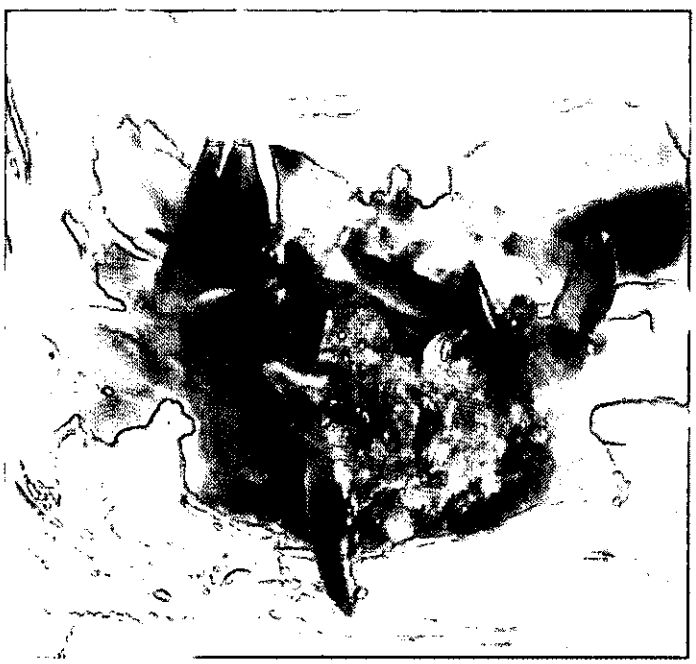

Figure 4.19 Vitrification of the shoots.

4.4.3 Seasonal Variation

The efficiency of the induction stage will vary throughout the year due to the climatic changes and the physiological status of the mother plants (Figure 4.20). It's been reported that induction is more efficient during the winter months when there is less light (Robert et al., 1992). Conversely, contamination is more recurrent during the rainy season.

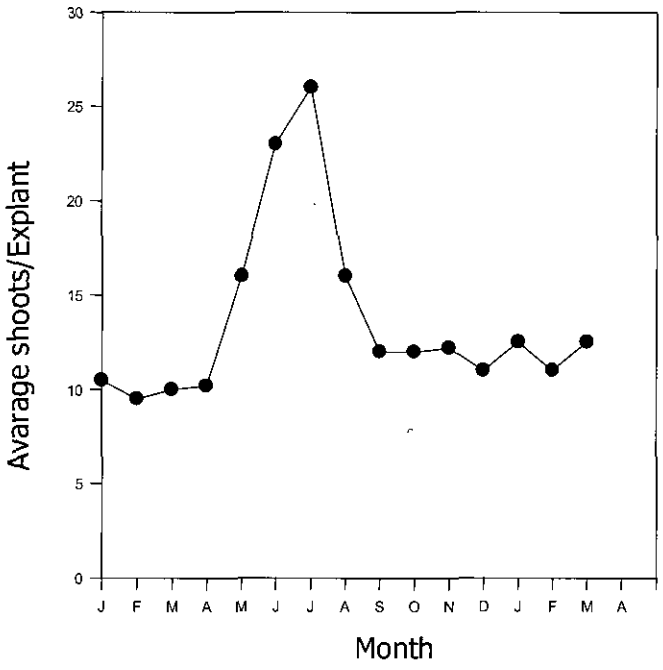

Figure 4.20 Seasonal variation in the induction of explants of *Agave tequilana* collected at different times of the year.

4.4.4 Intra and Inter Specific Variation

Different species will show some degree of variation in their culture requirements for propagation that will have to be satisfied to make the system as efficient as possible.

Very big differences are, however, observed between clones of the same species that are probably due to somatic variation within the cell populations. These variations are not always observed because they may be "hidden" and finally selected against and eliminated by other faster growing cells.

Table 4.1 *Agave* species and hybrids that have been successfully micropropagated through meristem culture and adventitious shoot production

Species	Variety	Origin of mother plant	Ploidy level	References
Agave amaniensis		Kenya, Tanzania	2x	CFC/UNIDO, 2004.
Agave Angustifolia.Haw	Bacanora	Sonora, México	2x	Esqueda et al., 2004.
Agave angustifolia	Chelem ki	Yucatán, México	6X	Robert et al., 2004.
Agave arizonica		Arizona USA		Powers & Backhaus, 1989.
Agave cupreata		Oaxaca, México		Puche, 2004.
Agave fourcroydes.Lem	Sak ki	Yucatán, México	5X	Robert et al., 1987.
Agave fourcroydes.Lem	Kitam ki	Yucatán, México	3X	Robert et al., 1987.
Agave parrasana		Coahuila, México.		Santa Cruz Ruvalcaba et al., 1999.
Agave potatorum		Oaxaca, México		Salazar et al., 2004.
Agave sisalana		Yucatán, México	5X	Robert et al., 2004.
Agave sisalana		Kenya and Tanzania	5X	CFC/UNIDO, 2004.
Agave sisalana	Hildana	Kenya	5X	CFC/UNIDO, 2004.
Agave tequilana.Weber	Azul	Jalisco, México	2X	Robert et al., 1992.
Agave victoria.reginae		Chihuahua, México.		Rodriguez Garay et al., 1999.
H 11648		Tanzania	2X	CFC/UNIDO, 2004.
H 11648	Kyriazi	Kenya	2X	CFC/UNIDO, 2004.
H1200		Kenya, Tanzania	2X	CFC/UNIDO, 2004.
Mlola 487		Kenya, Tanzania	2X	CFC/UNIDO, 2004.

Chapter 5

Experiments Recommended to Optimize the Protocols

Genetic Improvement

5.1 Introduction

5.2 Selection of explants and disinfestations method
- 5.2.1 Sources of explants
- 5.2.2 Disinfestation

5.3 Suggested experiments for the induction of explants and the multiplication stages
- 5.3.1 Concentrations of growth regulators
- 5.3.2 Culture media
- 5.3.3 NO_3/NH_4 balance and total nitrogen
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- 5.3.5 Gelling agent
- 5.3.6 Other components of the culture medium
- 5.3.7 Light and photoperiod

5.4 General recommendations
- 5.4.1 Continuous observation and record keeping
- 5.4.2 Photography
- 5.4.3 Quality control

Chapter 5

Experiments Recommended to Optimize the Protocols

5.1 Introduction

No matter how closely related they are or how similar they look, not all the species of the same group of plants will grow and develop as well under the same *in vitro* culturing conditions. One species might develop better in a different growth medium with a higher concentration of calcium, while another may require higher concentrations or balance of auxins and cytokynins. Another one might be particularly susceptible to the concentration of a certain vitamin or, simply, not like a gelling agent. This was plainly established in chapter 2 where it can be seen that different authors report different conditions to reach similar results with different species.

The purposes of the investigation or the production objectives might also require adjusting the culturing conditions of a certain species and, not less importantly, the personal styles and preferences of the scientists for a certain strategy also play a role in the way a protocol is established in each laboratory.

Although most of the *Agave* species that have been tested develop adequately under the conditions described in chapter 4, a laboratory trying to culture a specific line might want to vary the conditions in order to establish a more efficient propagation system. However, the relative benefits between cost and efficiency, and the specific conditions of the laboratory and the region where it operates must be kept in mind at all times.

5.2 Selection of Explants and Disinfestations Method

5.2.1 Sources of Explants

The selection of the source of explants might be the first variable that needs adjustment since offshoots from rhizomes might not be available or they might be very small and stressed (e.g. in plants collected from the dessert).

Mature plants are definitely not recommended since they are very fibrous and difficult to cut and, in general terms, are more infested with microorganisms than young rhizomes. But, even if they can be obtained,

it will be very difficult to induce the formation of new shoots from these tissues.

Bulbils are the natural alternative and the conditions for induction must not be very different than those recommended for rhizomes. However, probably only one explant will be extracted from each bulbil and this will require the extraction of many individual explants that will have to be directly disinfested.

Seedlings from germinated seeds are a suitable material for callus induction if a different morphogenic pathway is going to be followed but this should be considered a last resort as a source of meristematic tissue.

Other sources of somatic tissues such as axillary buds can be tried but it is not recommended since it will require an enormous amount of work and the probabilities of success are very limited.

If a different source of explant is used, other than rhizome offshoots, adjustments will definitely have to be made to the methods employed for disinfestations and induction since the small explants will be more directly exposed to the chemicals used.

5.2.2 Disinfestation

Irrespectively of the use of chemical biocides in the culture media (see chapter 7) the explants must be treated to eliminate microbial contaminants before they are placed in culture.

For this purpose it is always recommendable to define the concentration of the chemicals used and the times the tissues need to be immersed in them.

Sodium and calcium hypochlorite are the first choice, supplemented with a surfactant such as tween 20.

It is recommended to try a range of concentrations around 0.6 to 1.5% active chlorine.

The best treatment is the one that completely eliminates the exogenous superficial microorganisms (not the endophytic ones) producing the least damage to the plant tissues. Biocides such as PPM, Oromex and Vitrobaf can be a better option for small explants.

To determine the effectiveness of the treatment it is best to incubate the treated explants in rich microbial culture media (see chapter 7) that will rapidly show microbial growth. However, the effect of the treatment on the plant tissues will only be seen after they respond to induction producing new shoots.

5.3 Suggested Experiments for the Induction of Explants and the Multiplication Stages

The most important chemical and environmental variables that ought to be tested for induction and/or multiplication are the following:

- Growth regulators
- Composition of the culture medium
- Nitrogen balance
- Type and concentration of sugars
- Gelling agents
- Light and photoperiod

For experiments on induction:

- Use only the top level of the explant and discharge the rest of the tissue.
- All explants from different mother plants should be randomly distributed
- Place 4 or 5 explants/culture dish (baby food jars).
- Make a minimum of 2, preferably 3 replicates (8-15 explants/treatment) depending on the amount of plant material available.

For experiments on multiplication:

- Use homogeneous shoots (3 cm.) that have been cultured, for at least a couple of weeks, in magenta boxes kept under the same culture conditions in medium without growth regulators or any other substance that might mask the results.
- For each treatment use a minimum of three replicates of 20 shoots per magenta box.

5.3.1 Concentrations of Growth Regulators

Growth regulators, particularly the balance between auxins and cytokinins, are the most important factors to be considered for an effective micropropagation system.

First explore a broad range or concentration and interactions:

Auxins (mg/1)	Cytokinins (mg/1)			
	0	**5**	**10**	**15**
0				
0.1				
0.025				
0.05				

4 x 4 x 8 = 128 explants would be the minimum required for this experiment. Since the number of explants required might be too high for an initiation experiment, it is recommended that the number of interactions, rather than the number of replicates, is reduced.

Once there is a clear picture or the range of concentration that is optimal for a specific purpose the balance can be confirmed and fine tuned, as in the following example.

Auxins	**Cytokinins**		
	12.5	**15**	**17.5**
0.025			
.05			
.075			

The main auxins and cytokinins to be tested are:

Auxins: IAA, 2, 4-D and MECOPROP

Cytokinins: 6-BAP, kinetin, and TDZ

The final choice will depend on the numbers and the quality of the plants produced and, if results are similar, on the cost of the chemicals.

Other growth regulators such as ABA, gibberellins, antiauxins etc. should be considered only in cases of extreme necessity.

5.3.2 Culture Media

The most commonly used plant tissue culture media are all mixtures of macro and micro elements in the form of the most common salts supplemented with the basic vitamins and a sugar as a source of carbon. Amongst these the formulation designed by Murashige and Skoog (1962) known as MS has been the most widely used in spite of its high levels of nitrogen.

If the tissues do not grow well in MS, the same medium can be used at half its strength (½ MS) or other well defined culture media such as Schenk and Hildebrandt (SH) or B5 (see culture media compositions in Annex I).

The concentration and balance of the nitrogen ions can have a great impact on the multiplication capacity of the agaves and that led to the formulation of MS-B (Robert et al., 1987) which contains 10 mM KNO_3; 5 mM NH_4 NO_3.

5.3.3 NO_3/NH_4 Balance and Total Nitrogen

To optimize the levels of these ions it is recommended to try the following molar balances:

	KNO_3		
NH_4NO_3	0.75	1	1.25
0.75			
1			
1.25			

5.3.4 Type of Sugar and Concentration

The carbon source represents a high percentage of the cost of the culture medium. Sucrose is the most common carbohydrate used for plant cultures and it is also the cheapest. Highly pure sucrose is not really necessary and icing sugar and ordinary sugar locally available might be quite adequate, however, it can be highly variable and should always be tested and compared with a pure commercial (e.g. Sigma) sucrose sample at concentrations ranging between 10 and 30 g/l.

5.3.5 Gelling Agent

In semisolid cultures good gel support is very important because the quality of the plants depends on it and it is needed for culturing and easy manipulations.

The main gelling agents are: Agar, Gelrite (gellan gum) and Phytagel. They are all suitable for agaves but agar is by far the cheapest. The use of very pure agar might not be necessary and cheaper agars can be used but they should be thoroughly tested.

Sometimes, the concentration required for adequate gelling varies from one batch to the next even in the finest reagents, and this is something that must be checked regularly since a soft medium will not support the plants and will induce vitrification.

A mixture of agar and gel rite is recommended for the pre-adaptation stage to harden the media and reduce the water pressure of the gel.

5.3.6 Other Components of the Culture Medium

The concentration of a specific vitamin or an unusual compound might be critical for development but this should not be addressed unless there are serious difficulties with achieving adequate results.

5.3.7 Light and Photoperiod

Light quality and intensity are probably the most variable factors in any tissue culture protocol because they depend on the specific conditions of a particular laboratory.

Each culture room has a different setting with different types and numbers of lamps per shelf placed at different heights from the cultures. Culture dishes are incubated in one layer or on top of each other; some rooms have windows that allow natural light in etc.

It is important to find out the most adequate conditions for each stage and make the most of the conditions available and, if possible, modify them to suit the needs.

The induction of the first shoots (stage I) benefits from a few days in the dark before transferring the cultures to continuous light. However, multiplication proceeds better under a 16h photoperiod and pre-adaptation *in vitro* benefits from high light intensity (preferably natural light). It is convenient to decide if all of these changes that greatly complicate the process are strictly necessary or the different stages can be managed with simpler conditions even if they are not the optimal ones.

5.4 General Recommendations

Although the following points have nothing to do with the protocol itself, their observance is very important for this procedure to work properly; if necessary adjustments should be made.

It must be remembered that changes in the physiology and, therefore, in the performance of the cultures can arise at any moment due to aging, habituation or other causes and that this might call for adjustments.

5.4.1 Continuous Observation and Record Keeping

Continuous observation of cultures is very important in order to be able to detect the smallest changes that might start to take place, such as vitrification, or the appearance of microbial contaminants, or a decrease of the multiplication factor.

Keeping records is one of the most important aspects of tissue culture work. Without excuse or exception, all operations carried out during the day must be recorded in an official (registered) bound log book with numbered pages. Every single transfer must be labeled with a code that tells its full story from the beginning, indicating its origin, number of transfers and conditions and any additional information that might be useful. Productivity records to measure the overall efficiency of each clone, every technician and the laboratory as a whole are also needed. Someone must be responsible for all the record keeping.

5.4.2 Photography

The best way to record the state of mother plants, cultures, products, etc. is by means of images. It is very important that the technical staff learns to take good quality photographs, not only getting the right exposure but ensuring proper framing and focusing (depth of field) so that the pictures show exactly what is intended to be shown.

5.4.3 Quality Control

To ensure the highest quality and efficiency, absolute concentration must be kept throughout all procedures, particularly during cutting and sorting of plantlets. Routine and excessive confidence tends to result in a reduction in quality and, possibly, contamination.

Chapter 6

Transfer to Soil

6.1 Introduction

6.2 Pre-adaptation

6.3 Transfer to soil and weaning in the green house

6.4 Growth under shading

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Chapter 6

Transfer to Soil

6.1 Introduction

Probably the most critical stage of the whole micropropagation process is the moment when the plants leave the *in vitro* conditions to be transplanted to soil.

The main reason for this is the abnormal morphology and physiology of the plants that have spent months under totally abnormal environmental conditions. The environmental conditions in the culture dish are designed to promote rapid growth and development by providing the plants with large quantities of everything they need:

- High concentrations of auxins and cytokinins
- High concentrations of certain vitamins
- High relative humidity
- Low light intensity with altered photoperiods or darkness
- Protection against pests and disease

Transfer to soil implies that suddenly, after weeks of protection, the plants have to "adapt back to normality", something for which they are not well prepared. Most *in vitro* grown plants:

- Lack epicuticular waxes
- Have abnormal, non functional stomata
- Do not carry out photosynthesis
- Lack true roots

All of these features make them extremely susceptible to desiccation and, consequently, they suffer great stress when transferred to soil in open air. The survival and performance of the micropropagated plants will therefore depend on how well they are handled and adapted before they are transplanted to soil.

The importance of this stage must not be underestimated since it is the most critical for the survival of the plants and because any losses suffered at the end of the micropropagation process imply a corresponding economic loss.

Additionally, the future performance of the plants in the field will also depend on how well the plants are managed at this stage. The vigour that the plants show while growing *in vitro* must be maintained. The plants must not suffer any unnecessary stress that will slow or even arrest their development.

The adaptation to *ex vitro* conditions includes four stages:

- *In vitro* rooting and pre-adaptation
- Adaptation under high humidity in green houses
- Growth under shading
- Transfer to nurseries in the open

6.2 *In vitro* Pre-adaptation

The last stage of the *in vitro* culture process is designed to prepare the plants for life outside the culture dish. The formation of roots (Figure 6.1) in a culture medium without growth regulators (or with small amounts of auxin) is the main objective. However, if possible, this last transfer should be carried out under chemical and environmental conditions that will also favour hardening as was previously discussed in section 4.3. That will permit the initiation of new leaves, with less abnormal morphological and physiological characteristics, which will develop more rapidly and will help the plant to suffer less stress when taken out of the culture dish.

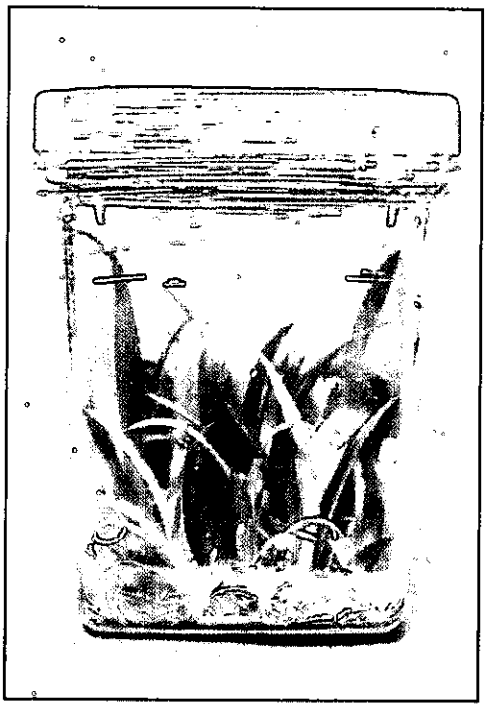

Figure 6.1 Rooted *Agave angustifolia* Haw. Plants ready to be transplanted to soil.

6.3 Transfer to Soil and Weaning in the Green House

This is the moment when the vitroplants start their life out of the culture dish and begin their development into mature plants.

The plantlets are taken out of the culture dish (Figure 6.2) and washed gently in running tap water to remove any residues of agar and/or sugar.

This is particularly important since sugar will favour the growth of fungi that can kill the plantlet.

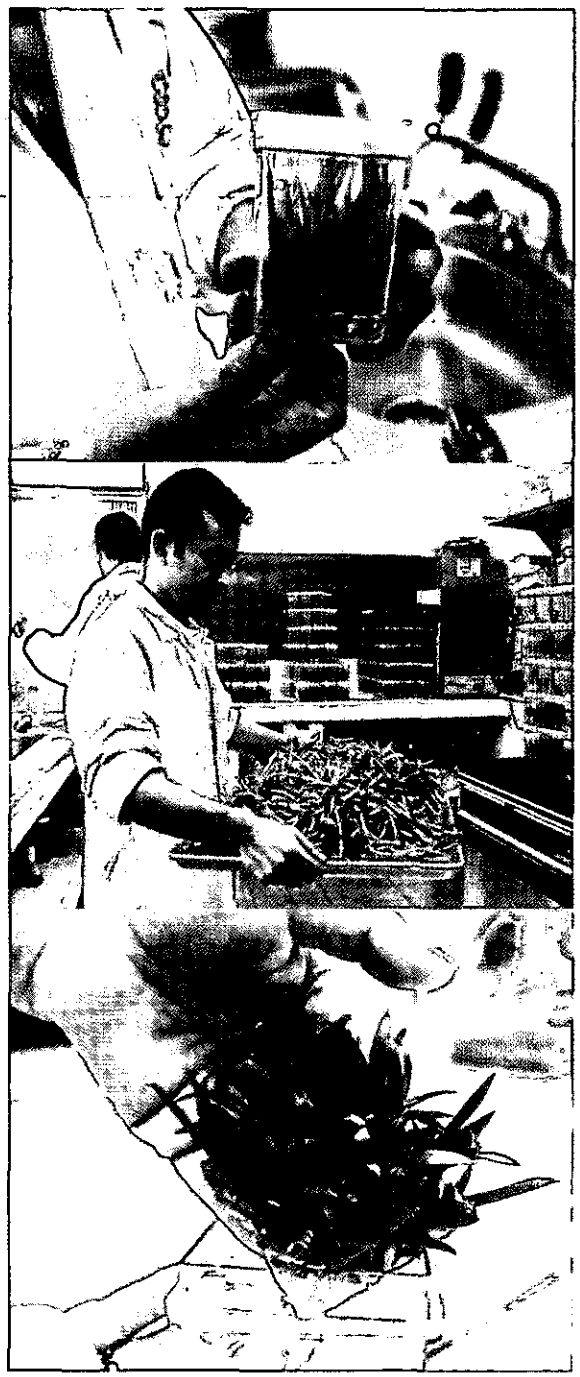

Figure 6.2 Rooted plants taken out of the culture dishes are washed and separated.

It is of the greatest importance that only fully grown plants are taken out of the *in vitro* culture containers. If the plants are smaller than 5 cm they should be transferred to medium without growth regulators for the length of time needed for them to reach the right size.

Plants transferred to soil in polystyrene trays must be of the same size (a minimum of 5 cm.) and have a minimum of three leaves (Figure 6.3a).

Before planting, the plantlets must be immersed in a solution of fungicide for a couple of minutes (see Annex II) to protect them from fungal contaminants.

The trays (200 wells) are filled with a mixture of pasteurized peat moss (Sungro Horticulture Canada LTD) 40% and soil (levisol) 60% and dampened before the plantlets are sown.

These trays are then placed in a green house (Figure 6.3b) at 30 ± 2°C under shading (200 to 400 µmole/m^2 /sec) with a 16h photoperiod and high humidity (>80%) for as long as necessary (about 1 month). These conditions have worked well for the adaptation of *Agave fourcroydes* Lem. with a survival rate of >95%, but could vary for other *Agave* species.

During this period the plants are watered regularly although the periodicity will depend on the time of the year and the prevailing environmental conditions. The watering should be gentle; if there is no mist installed, manual spraying should be applied always making sure that the substrate does not get too humid because that will lead to the rotting of the base of the plants.

Regular supervision to eliminate any weeds that might appear and to control pests or disease is strongly recommended since at this stage the plantlets are extremely susceptible to attacks from these sources.

The physical facilities to achieve these or similar conditions can be very variable and will depend on the actual facilities of the laboratory and the climatic conditions of the region where the plants are being produced. The range of options available is discussed in chapter 11; however, the construction of humid chambers inside the green house to maintain high humidity is an efficient and relatively cheap option.

Hot and dry climates, such as the ones in Baja California Sur and Sinaloa in Mexico, where the summer temperatures reach above 40° C make the adaptation of *Agave angustifolia* Haw and *Yucca valida* very difficult and it is sometimes necessary to maintain the plants indoors with air conditioning. In cases like this, the pre-adaptation stage becomes particularly relevant.

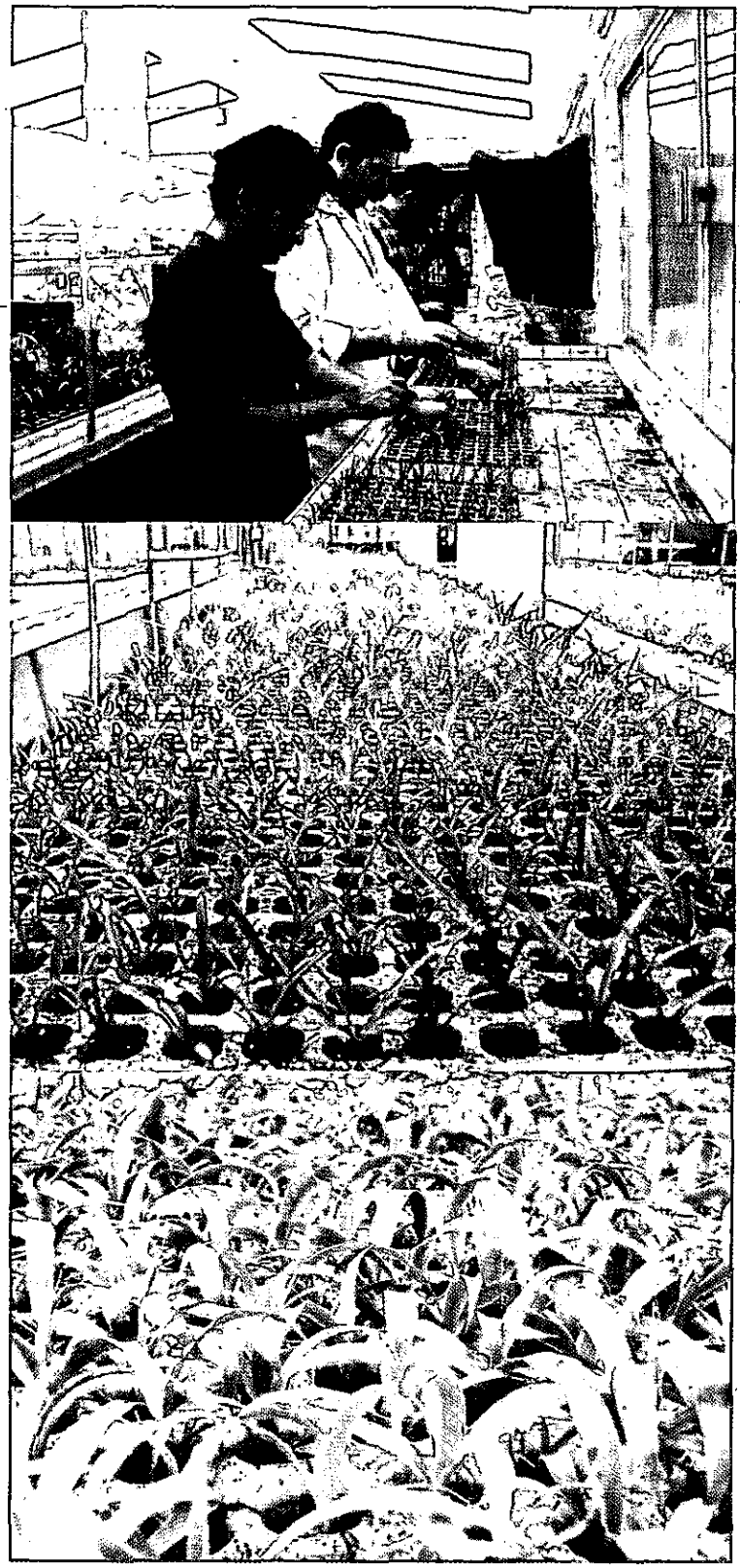

Figure 6.3 Vitroplants planted in polystyrene trays and incubated in a greenhouse with high humidity.

6.4 Growth Under Shading

After a month, the plants are ready to be moved to dryer conditions but must still be protected from direct sunlight. The trays should therefore be transferred to shaded areas in the open for another month or two (Figure 6.4). After this the plants should have a minimum of 5 normal leaves and should have reached a robust size, depending on the species being propagated.

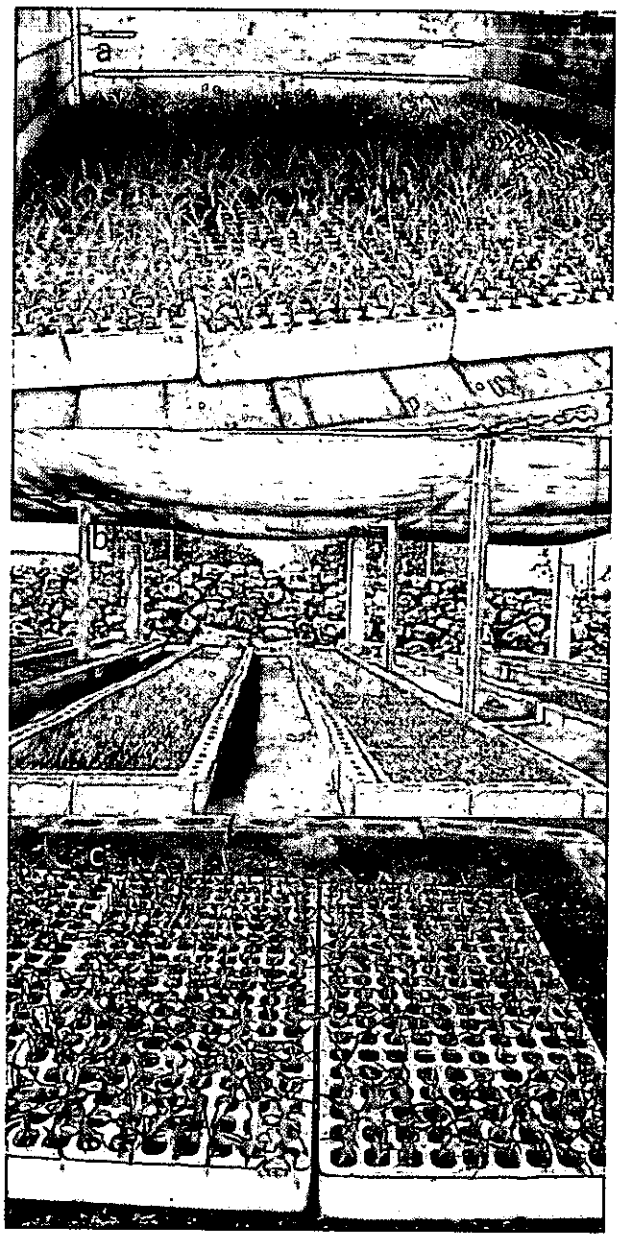

Figure 6.4 Trays are transported to planting areas (a) and placed under shading (b) on a bed of soft soil substrate (c).

The trays are placed in areas shaded with nets (70% shading) on 10cm high beds (Figure 6.4c), prepared with a fresh and well aerated local substrate (henequen bagasse) that allows the roots to continue growing through the base of the tray. If this is not the case, the growth of the

roots, and that of the whole plant ceases. As a general rule, the plants must remain in the trays for the shortest possible period of time since the constraining space in the wells will limit their development.

Watering by hose pipe with a sprinkler should be carried out every other day taking all the appropriate phytosanitary measures (see AnnexII).

The leaves formed *in vitro* are likely to dry out and their function will be to help the plant survive in the new environmental conditions. The new leaves, formed *ex vitro,* will be normal thick leaves, with normal functional stomata and a glaucous appearance, indicative of wax deposition on their surface and will continue growing throughout the whole time in the nursery and even into the early plantations stages. Depending on the species, marginal spines may or may not form.

Figure 6.5 Plants ready to be transplanted to nurseries.

6.5 Transfer to Nurseries

The adapted plants are then ready to be transplanted to nurseries where they will continue their development before they are taken to the definitive plantation sites.

Only those plants that have a minimum height of 10 cm and 5 leaves are removed from the trays by pulling them out (Figure 6.5) and planted on beds of organic matter (Figure 6.6). Although the exact way of making nurseries is a matter of convenience and tradition, in experimental henequen nurseries the beds (90cm.x 20m) are made of bagasse leaving a street 1m wide between them. This allows a planting distance of 30cm between plants and 30 cm between rows for a final density of 60,300 plants per hectare. Under these conditions, in 10-12 months the plants reach a height of 40-50 cm (compared to the 12-18 months required by traditionally propagated plants) and are ready to be moved to the definitive plantation sites.

Another big advantage of micropropagated henequen plants is the fact that they produce large numbers of rhizomes in the nursery (Figure 6.7), hugely increasing the numbers of plants propagated per clone and, consequently, reducing the costs of the whole micropropagation process.

Figure 6.6 Experimental and production nurseries in Yucatán (a and b) and ARI Mlingano, Tanzania (c).

6.6 Field Testing of Micropropagated Materials

Vigorous plants transplanted to the nurseries will grow well and will show the full potential of micropropagated plants when compared with their naturally propagated counterparts, whether they are bulbils or off shoots from rhizomes.

To keep control of the quality and performance of the micropropagated plants, experimental nurseries and plantations must be established in suitable locations where they can be supervised and attended at all times (Figure 6.8).

The work must be done in close collaboration with growers who should also evaluate the materials and compare them with their own.

Figure 6.7 New plants from rhizomes produced in the nurseries.

The plots must be planted under the same conditions in which they will be cultivated by the growers, except that the plants must not be stressed unnecessarily leaving them in the sun for days but planted as soon as possible.

It is recommended that, as a quality control, a small sample of the same lots of micropropagated plants are planted at the research stations or laboratories where they were produced.

6.6.1 Controls

To test the performance of micropropagated plants in the field it is recommended that they are compared with adequate controls. Ideally, plants of equivalent age or size, either from bulbils or shoots from rhizomes, should be planted side by side at the same time.

6.6.2 Measurements

A statistically significant number of randomly distributed plants should be measured at the moment of planting (time 0) and then every two months. Plant height and total number of leaves are sufficient at the nursery level. Diameter of the stem and the length and width of a middle leaf (3rd rosette) must be measured at the plantation stage.

6.7 Further Selection

Iirrespective of the way in which genetic variability is generated, all new materials require field testing. Those plants that show useful traits, derived from new combinations or mutations, can then be selected for further crosses.

Genetic improvement fixes new advantageous characteristics through a process of repeated selection. This is also true in the case of micropropagated plants. Once in the nursery or the plantation, it is important to continue careful observation in order to detect odd or outstanding traits. The selected plants must be marked, measured, photographed, and cared for so that their rhizome offshoots can be collected to be cloned again. The new clonal lines will go back to the field for further evaluation.

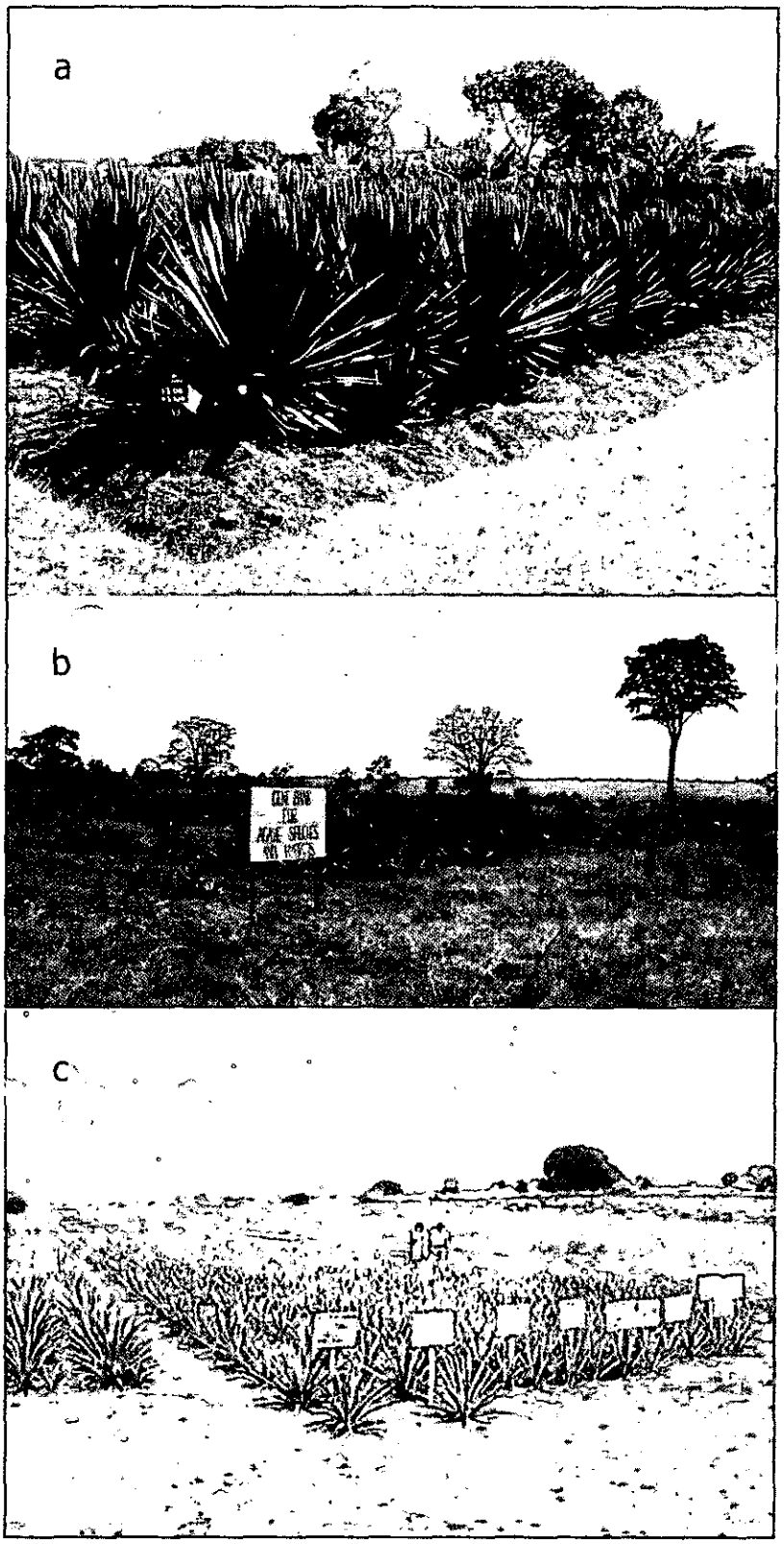

Figure 6.8 Experimental plots of micropropagated plants at a) Kephis Muguga, Kenya, b) The germplasm collection at ARI Mlingano, Tanzania and c) Tequila Herradura, México.

MTC Laboratory, ARI Mlingano, Tanga, Tanzania

Chapter 7

Control of Microbial Contamination

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7.2 Plant-microbe interactions

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- 7.3.2 Bacteria

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Chapter 7

Control of Microbial Contamination

2.1 Introduction

Microbial contamination is the most serious and frequent problem that affects *in vitro* plant tissue culture. It affects all species at all stages of the process, reducing its efficiency or producing serious losses. It can also be one of the most difficult to tackle due to the fact that microbes are everywhere and practically all of them can grow in the sugar-rich plant tissue culture media (Figure 7.1).

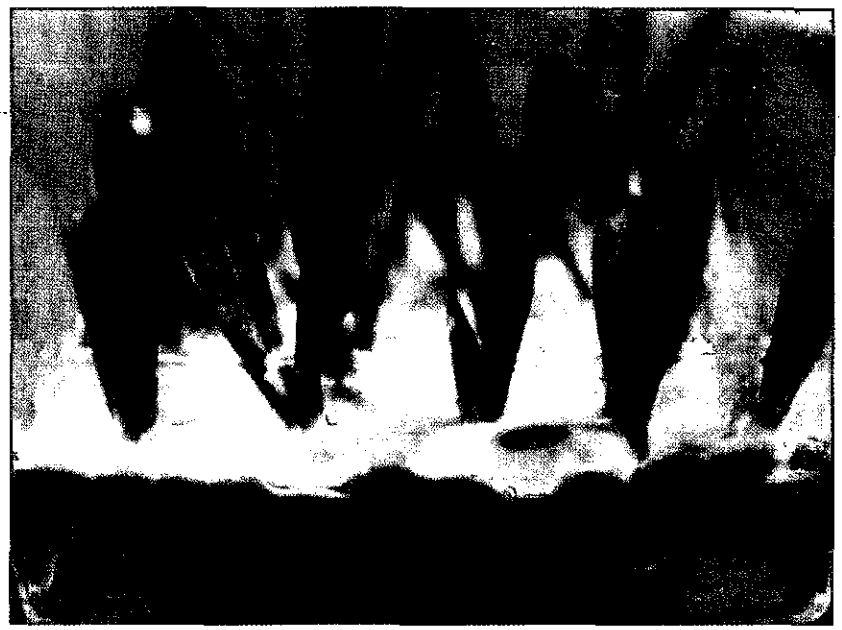

Figure 7.1 Culture contaminated with fungi

Tissue culture laboratories can suffer severe losses due to microbial contamination. This is often due to an inadequate phytosanitary supervision of the production systems, a poor understanding of the plant-pathogen relationship and an untimely implementation of preventive phytosanitary techniques (Leifert & Woodward, 1998).

Many important diseases are inadvertently dispersed by humans through the movement and planting of infested materials or plant products that have not been adequately monitored. This is particularly true under the present trends

of open global markets in which the movement of plants without the appropriate phytosanitary controls can produce severe economic problems. In consequence, the elimination of the microorganisms that accompany the plant tissues becomes one of the most critical steps of the whole *in vitro* culture procedure.

Tissue culture has been heralded as a method of producing disease-free plants for commercial crops and one of its most important applications is that it offers the safest mechanism to move germplasm between different geographic areas; however, if used in a careless manner, tissue culture can, instead, become a mechanism for disease dispersal.

Dealing with microbial contamination requires more than the simple application of preventive or corrective measures, it requires a basic understanding of the nature of the problem and the implementation of integral strategies to deal with it. In this chapter a brief analysis is presented of the main factors that must be considered for the phytosanitary control during the micropropagation of agaves and recommendations are made regarding the methods that should be applied to ensure the control of microbial contamination in the *in vitro* culture process.

7.2 Plant Microbe Interactions

In nature, plants and microorganisms coexist with different degrees of interactions that go from **neutral** interaction, a simple physical, not metabolic co-existence, through harmless **commensalism** (epiphitism) where one of the organisms benefits from the interaction without harming the other one, and **mutualism** in which there is a mutual benefit for both species, to **parasitism** in which the microorganism lives off the plant tissues, damaging them and even killing the plant (Robinson, 1987; Baker et al., 1997).

In the natural undisturbed ecosystems, the populations of plants and microorganisms are in equilibrium with each other. However, when this equilibrium is broken and one plant species takes predominance on all others, as occurs in agricultural systems, the growth of the microbial populations is also altered (Scheffer, 1997).

Something similar happens when plants are cultured *in vitro* where there is only one species growing in an artificially rich and stable environment which is particularly favorable for the proliferation of microorganisms: excess of nutrients, mainly carbohydrates, high humidity and reduced light intensity promote the growth of bacteria and fungi that outgrow the cultured plant

tissues, causing severe damage and producing heavy losses that render the micropropagation process inefficient and more expensive.

Microorganisms can live on the surface of the tissues, in the intracellular spaces or even inside the plant cells. These endophytic microorganisms, not necessarily phytopathogenic, are the most difficult to eliminate and the ones that can present the greatest problems for plant tissue culture (Thompson et al., 1993; Marcell and Beattie, 2002).

7.3. Microorganisms Associated with Agaves

The agaves, as all other plants, interact with various species of microorganisms such as viruses, bacteria and fungi. These interactions can be neutral, mutualistic such as the case of the mycorryza formed in the roots of most agaves (Armenta Calderon, 2002) or parasitic.

Very little is known regarding the nature of the pathogens that cause diseases in agaves since no conclusive identification has been made for many of them and it is not even clear if the diseases with similar symptoms reported for the agaves of different parts of the world are the same disease, caused by the same pathogen, or not.

7.3.1 Fungi

Williamson et al. (1998) carried out a study of the main fungal contaminants in public and private micropropagation laboratories and found the following genera in order of importance: *Botrytis, Pythium, Rhizoctonia,* Downy mildews, *Phytophthora,* Powdery mildews, *Fusarium,* Brown mould and *Penicillium.* As it can be observed, all of them are pathogenic fungi although some strict saprophytes were also reported.

The pathogenic fungi reported to be responsible for known diseases in the agaves are summarized in Table 7.1. At least two genera (*Phytophthora* sp and *Colletotrichum* sp) could be considered endemic since they have been isolated from different regions and in different species. *Penicillium* and *Aspergillus* are also frequently found as contaminants of *in vitro* cultures (Quijano et al., unpublished data).

7.3.2 Bacteria

More than 200 bacterial species have been reported to induce diseases in plants and so far only four species of phytopathogenic bacteria have been reported in *Agave* species (Annex III). The first one: *Pantoea agglomerams* induces a soft rot in the stem (pseudostem) of *Agave tequilana* (Jimenez-

Hidalgo, 2004). The symptoms of this disease are similar to the disease known as bole rot, reported for the agaves cultivated in Africa (Tanganyka Sisal Grower´s Association, 1965).

Dry leaf tip (Quijano et al., 2004), which resembles tip dieback in Africa, is caused by an interaction between *Pantoea agglomerans* and *Enterobacter cloacae.*

Finally, the disease known as shotgun, because of the round hole lesions it produces, is caused by *Enterobacter cloacae.* Saprophytic bacteria belonging to the genera *Bulkhoderia and Psedomonas* were also found to be present in the leaves of *Agave fourcroydes* (Quijano et al., 2004*).*

7.4 Establishment and Maintenance of Aseptic Cultures

The production of healthy, high quality materials is one of the main reasons for culturing plants *in vitro* and every effort should be made to ensure that the plants produced are free of disease, so special care must be taken with any known pathogenic species, whether from agaves or other species.

There are two types of contaminants that need to be dealt with in the plant tissue culture systems:

- the ones that are present in the explant at the time the cultures are initiated (endogenous contamination) and
- those that are inadvertently introduced during the manipulations of the tissues (exogenous contamination).

The endogenous contaminants need to be eliminated before the tissues are established *in vitro* while the second type needs to be prevented from entering the culture systems once they have been initiated.

In the case of the first, several tissue disinfestations steps should be followed to ensure that no microorganism is present when the explant tissues enter into direct contact with the sugar-rich medium (see section 4.3.5). In the case of the second, preventive actions are needed to block the entry of contaminants to the laboratory and to the culture dishes (see section 7.8)

7.5 Detection of the Presence of Microbial Contaminants in Plant Tissues

The indexation and identification of microbial contaminants in plant tissues cultured *in vitro* are very important practices that are seldom implemented in tissue culture laboratories because this implies more work and higher

production costs. However, they should be compulsory in cases when it is known that there are specific phytosanitary problems associated with a crop in a specific region or when there are recurrent contamination problems with *in vitro* cultured tissues.

Although microorganisms present in the explants sooner or later manifest themselves in the cultures, some microorganisms might remain latent or inhibited by something present in the plant culture media and only start growing when the cultures get old or are incubated for long periods of time.

The presence of endophytic bacteria, living inside the cells, will not be immediately obvious and might emerge at any moment after the cultures have long been established (Figure 7.2). Although most of these bacteria are completely innocuous for the plant tissues, they can still ruin a micropropagation process if they start growing. It is therefore convenient to be able to detect their presence right from the beginning and, if possible, to eliminate them before the cultures are initiated (Kamoun et al., 1998, Kritzinger et al., 1998).

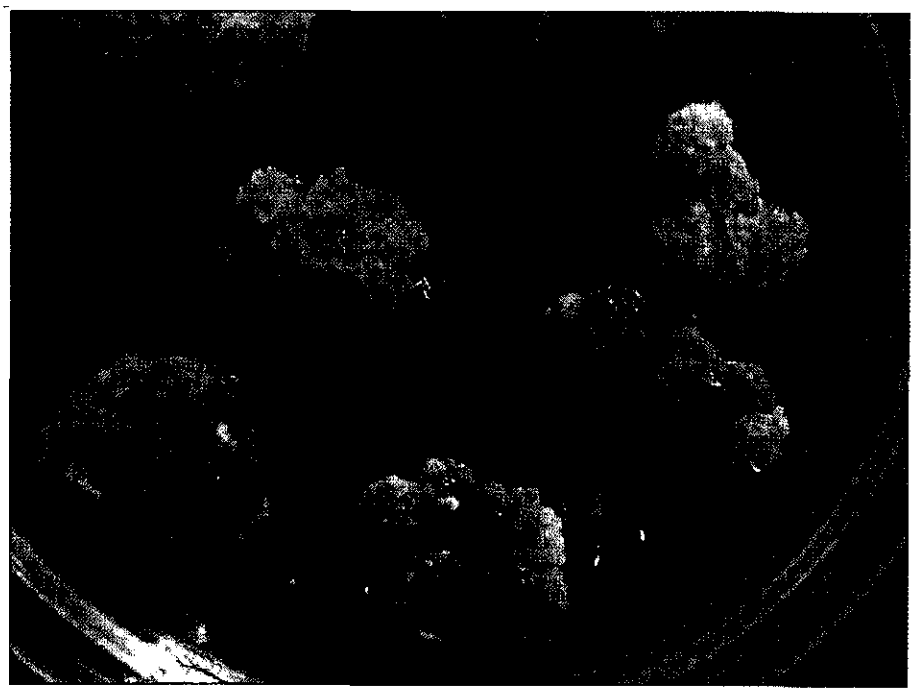

Figure 7.2 Localized endogenous bacterial contamination on a callus tissue.

7.5.1 Culture in Liquid and Semisolid Media

The easiest way to detect the presence of microbial contaminants in the plant tissues to be cultured is to incubate sections of these tissues in different culture media designed to promote the growth of all sorts of microorganisms.

After washing and chemical disinfestation, the tissues are cut into small pieces or macerated and incubated in liquid culture media or planted on semisolid agar media. The presence of microorganisms becomes manifest after 48 to 72 hours (Figure 7.3).

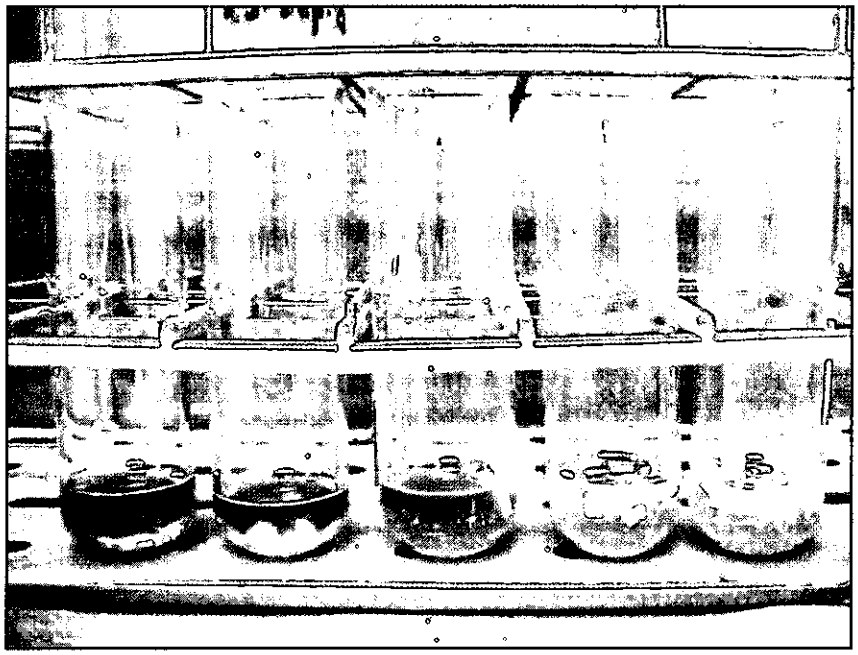

Figure 7.3 Microbiological cultures to detect the presence of endophytic bacteria in plant tissues before culturing them. Turbidity in the culture media indicates the presence of microorganisms.

7.5.2 Protocol for the detection of microbial contaminants in cultured tissues

- This process must be carried out under totally aseptic conditions to make sure that the only microorganisms detected are those present in the tissues.
- Pick randomly five plants from the culture dishes.
- Macerate the tissues with a sterile glass rod bar in sterile distilled water.
- Take 1ml of macerate (including bits of tissue) and add it to a test tube with 9ml of nutrient broth (Sigma Chemical Co. Cat. N7519) supplemented with 0.5% yeast extract and 0.1% glucose.
- Seal the tubes and incubate them in darkness at 28°C with continuous shaking (100 rpm) for 72-120 hours.
- The presence of microorganisms will become manifest by the appearance of turbidity (Figure 7.3).
- To corroborate the presence of microorganisms and to separate the putative contaminants, 250 µl of the culture are plated on a Petri dish

containing Screening bacterium medium (Sigma chemical Co. Cat. B-1662). To prepare the dishes follow the manufacturer's instructions.
- Incubate the Petri dishes in the dark at 28°C for a period of 48-96 hours until clear colony plaques appear on the surface of the medium.

For identification, the colonies must be re-isolated and sent to a laboratory that can do the necessary tests (Annex III).

7.5.2.1 *Detection of environmental contaminants*

- Place open plates with enriched culture medium in the areas to be screened (air flow cabinets, growth rooms, etc.) for 24 hours.
- Incubate as indicated above.

7.6 Techniques for Decontamination

7.6.1 Meristem Culture

Meristems are the regions of tissue where cells are undifferentiated and in active division. They are the points where growth takes place whether it be to produce enlargement (apical meristems), thickening (cambium) or to produce new branches or flowers (axillary meristems).

Since the meristematic cells are undifferentiated, no vascular tissue is present in these regions and that might be the reason why no microorganisms are present in them. This means that the *in vitro* culturing of these tissues will generate plants that are free from microbial contaminants. This characteristic has been used to produce axenic plants of many species (Mateja et al., 1998; Gurr et al., 1992).

The culture of meristematic regions is not always simple because it implies isolating and culturing a very small portion of tissue; most apical meristems are not larger than five layers of cells and this requires very delicate manipulations that must be carried out under the microscope. Furthermore, culturing such small pieces of tissue often requires special care, since the cells might easily get damaged or desiccated.

Finally, culturing the meristems does not always guarantee that the new plants will be free of microorganisms and complementary treatments may be required to fully eliminate all the contaminants from them (Mateja et al., 1998).

7.6.2 Thermotherapy

Stressing the plant tissues at relative high temperatures (42-45ºC) for short periods of time can eliminate microbial contaminants from some plant tissues. Although this treatment is used mainly to eliminate viruses from explant tissues before they are cultured *in vitro*, Longens-Gerrits and colaborators (1998) reported that they were able to reduce the presence of bacteria in *Lilium speciosum* bulbils by using thermotherapy where treatment with sodium hypchlorite had not been successful. By immersing the bulbils in warm distilled sterile water for 1 hour they achieved an effective reduction from 50 to 5% without significantly affecting the redifferentiation capacity of the tissues.

7.6.3 Chemical biocides

One of the most effective ways to control contamination is through the incorporation in the culture medium of certain chemicals or mixtures that have a strong antimicrobial activity but that do not significantly affect the growth of plant tissues. Among the most widely employed commercial products are:

PPM (plant preservative mixture) is an undisclosed mixture of chemicals with a broad spectrum biocide activity (see Annex II) that is used for the control of microbial contamination of plant tissues cultured *in vitro*. It is not toxic (at the recommended doses) and does not affect growth or the morphogenic capacity of the tissues. Also, it does not generate resistance in the microorganisms (Niedz 1998). It is very effective preventing the exogenous contamination of bacteria, fungi and yeast but it does not eliminate endophytic bacteria (Babaoglu and Yogancilar 2000).

In *Agave fourcroydes* a final concentration of 0.2% of PPM effectively suppresses the growth of microorganisms with no significant effect on the performance of the cultures.

A similar substance is **OROMEX (**5-cloro-2-metil-4 isotiazolin-3-ona y 2-metil-4-isotiazolin-3-ona) a powerful biocide very effective in the control of fungi, bacteria and viruses at concentrations as low as 50 to 400 ppm. It is biodegradable but resists autoclaving. It has been used to control the growth of all types of microorganisms but must be handled with care because at 1.5% it is corrosive and can cause damage to the eyes and skin. It can be purchased from Plant Cells Marketing S.A. de R.L. y C.V. (see Annex II).

Another useful product is **Agrosyn** (Plant Cells Marketing), a superoxigenated water with a slight chlorine and ozone odour. It is used in medicine to treat infected skin wounds and, since it does not irritate the skin, it can be used for the continuous sterilization of tools in the air flow cabinets (without heat).

In tissue culture laboratories it is used to soak mats at the entrance and wash walls and working areas.

All of these products contribute significantly to control microbial contamination in all types of plant cultures and species but they are very expensive.

7.6.4 Antibiotics

The use of antibiotics to control contamination *in vitro* is not recommended because of their:

- high cost
- high toxicity to plant tissues and
- lack of effectiveness against fungal (eukaryotic) contamination

Before any antibiotic is applied to a tissue culture system, bacterial isolations and antibiograms should be carried out to be sure of their effectiveness. The degree of toxicity on the plant tissues must also be determined prior to use.

7.7 Techniques for the Identification of Microbial Contaminants

The techniques to index and identify microbial contaminants vary in their degree of complexity and cost and cannot all be carried out in all laboratories. However, the simplest techniques to detect the presence of microbial contaminants should be implemented as a routine in all tissue culture laboratories, leaving the identification to specialized ones that can do the job for a small cost (Annex III).

7.7.1 Use of Nutritional Kits

This method is based on differences in the biochemical and physiological characteristics of different bacterial species which are capable of metabolizing specific substrates but not others. As a consequence, each species can be identified by its ability to grow on certain culture media (observed as turbidity in the culture media) but not in others, or to react with certain substrates (producing colour) but not with others. The pattern of positive and negative reactions to a battery of tests permits a quick and simple, although rough, identification of the species.

There are two widely used commercial kits for microbial identification: that can be purchased from Biolog Inc. USA and Bioumeriux (St Louis Mo.).

7.7.2 Fatty Acid Profile

This method, based on the composition of fatty acids present in the bacterial cell walls, generates very useful taxonomic complementary information due to the great degree of variability and characteristic patterns of these molecules in each different species. The most widely used commercial system is the one from de MIDI (Newark, DE, USA).

7.7.3 Polymerase Chain Reaction (PCR)

This technique is much more precise but requires specific equipment and certain degree of expertise in molecular biology techniques. It is an extremely sensitive technique because it can detect the presence of a single cell and make thousands of copies of its DNA that are then used to sequence specific regions of the genome for comparison with known sequences present in the Genebank database. Therefore, it is mainly used to identify species that have been previously isolated and identified and whose genome has been partially sequenced and stored in such data banks (Louws et al., 1999; Henry, 1997; Stead et al., 1998).

7.7.4 Amplified Fragment Length Polymorphism (AFLP)

This technique permits the comparison of band patterns formed on an agarose gel by electrophoresis of the DNAs from different species that have been cut by restriction enzymes. The patterns are very specific and permit an accurate identification by comparison with the pattern formed by the DNA of known species.

The use of one technique or another will depend on the objectives of the micropropagation process. If the objective is to control the level of contamination of the process, the simple methods will suffice, but, if the objective is to produce plants completely free of a specific pathogen, the more accurate ones will be necessary (Henry, 1997 ; Gurr, 1992).

7.8 Prevention of Microbial Contamination in the Laboratory

Microbial contamination is the main enemy of efficient tissue culture and it is a problem that might emerge at any moment during the life of a micropropagation laboratory, causing serious losses. The best way to prevent contamination occurring after the cultures have been adequately established is to maintain discipline and absolute cleanliness in the laboratory.

Contamination of *in vitro* cultures can arise from airborne contaminants that get into the laboratory through doors or air conditioners or in peoples'

clothes. Once in the laboratory, microorganisms can get into the culture dishes during the manipulations or incubation at all stages of the process. To avoid this occurring, the following aspects regarding the structure and operation of the laboratory should be considered:

7.8.1 Laboratory Cleanliness

- Absolute cleanliness of all areas is essential and limited access to the working areas is convenient.
- Check for any leaks of air and/or water into the laboratories and seal them with silicone.
- Clean all walls, shelves and equipment with strong antimicrobial chemicals such as dettol (4.8% 3,5-di-choroxylenol) locally, putting special attention to edges and corners that are never cleaned.
- Floors must be cleaned thoroughly, removing the dirt and not just swept with a wet cloth.
- Air conditioners can also be a source of contamination. Every part of the air conditioner should be cleaned with dettol and ethanol at least every 3 months. The filters and all external parts or air conditioners should be regularly sprayed with an antimicrobial solution.
- Use the UV sterilizing lamp at night to maintain sterility in specific areas. The lamp *must not* be used where personnel or tissues are present.
- Additionally, more stringent methods could be implemented, such as limiting even more the access to the culture areas.
- Special shoe ware should be available only to be worn in the clean areas.
- Clean lab coats must be used at all times and the use of mouth masks and head covers is recommended.
- Air flow cabinets must be checked periodically by a specialized technician. This, however, will be very difficult in some parts of the world and it is therefore recommended that Petri dishes with microbial culture media are regularly placed open overnight inside the airflow cabinets to detect contaminants, and the pre-filters are washed or changed regularly.
- Sterilization should be checked by the use of indicator tapes and culture dishes must be pre-incubated for a few days before used, eliminating those that show contamination.

7.8.2 Manipulations

- Hands and arms must be thoroughly washed before working at the air flow cabinets.
- Air flow cabinets must be thoroughly cleaned before and after use and should be left running for at least half an hour before new materials are introduced. Air flow cabinets and culture rooms must be monitored

regularly for the presence of microbial contaminants by exposing open culture dishes with a rich microbial culture media for periods of 15-30 min. If bacterial colonies result, then the filters should be changed. The pre-filters should be washes every month.

- Special care must be maintained during sterilization and in all manipulations carried out within the air flow cabinets to avoid relaxation of good clean practices.
- The most important recommendation in this section is: never use the same tools to handle different clones or plants from different culture dishes. More forceps and scalpels should be acquired so as to prevent cross-infections when handing cultures in the laminar flows.
- Place cotton strings at the edge of the tops of the culture dishes and wrap the tops with commercial wrap (this will also help prevent desiccation of the culture media).
- Check for microbial contaminants before starting induction, incubate explants on growth media for 48 hours (preferably on rich microbiological media) and discharge any contaminated materials.

Table 7.1 Fungal and bacterial species that have been reported as phytopathogens in the genus *Agave*.

	Causal agent	Disease	Species	Reference
Fungal pathogens	*Colletotrichum agaves*	Antracnose	*A. sisalana*	Tanganyka growers association, 1965
	Phytophthora sp	Zebra disease	H 11648	Tanganyka growers association, 1965
	Phytophthora sp	Bole rot	*A. sisalana*	Tanganyka growers association, 1965
	Colletotrichum sp	Antracnose	*A. fourcroydes*	Diaz-Plaza, 1986
	Phytophthora sp	Bole rot	*A fourcroydes*	Diaz-Plaza, 1986
	Cercospora sp	Dry rot pole	*A fourcroydes*	Diaz-Plaza, 1986
	Tielaviopsis paradoxa sp	Soft rot	*A tequilana*	Fuchickovski, 2002
Bacterial pathogens	*Pantoea agglomerans* *Pseudomonas* sp	Sofá rot	*Agave tequilana*	Jiménez-Hidalo et al. 2004
	Pantoea agglomerans *Enterobacter cloacae*	Leaf dry tip	*A fourcroydes*	Quijano et al 2004.
	Klebsiella sp	Bole rot	*A fourcroydes*	Quijano et al unpublished data
	Enterobacter cloacae?	Shot gun	*A fourcroydes*	Quijano et al unpublished data

Chapter 8

Design of a Large Scale Production Scheme

8.1 Applied micropropagation

8.2 Large scale protocols

8.3 Design of a large scale production line

8.4 Flow chart for production control (an example)

8.5 How to use the flow chart
- 8.5.1 How many plants can be produced?
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Chapter 8

Design of a Large Scale Production Scheme

8.1 Applied Micropropagation

Most scientific laboratories that work on micropropagation techniques never take aspects such as cost and efficiency into consideration. The objective of their research is to publish scientific papers that report methods to propagate plants *in vitro* without any reference to the efficiency and economics of the process.

Applied micropropagation, however, goes beyond the research laboratory; it is a commercial enterprise that has to be managed as an industrial production line in which effectiveness and efficiency are paramount because they mean more or less profit.

Large scale micropropagation is the way in which tissue culture becomes applicable. It implies the production of thousands and thousands of plants for agricultural, horticultural and forestry purposes that have little to do with publishing. In fact, in most cases, commercial laboratories ignore the scientific literature and establish their protocols on the basis of their own experience.

It is an activity that must be run by a proficient scientist but administered by a commercial manager.

At present, there is a tendency for the work of many publicly funded scientific laboratories, mainly in developing countries, to become increasingly oriented to producing applied results that can be transferred to both the public and private sectors. In such cases the research must be oriented, right from the beginning to produce methods that can be commercially applied.

Some times research laboratories sign contracts, mainly with private industry, that imply the production of a large number of plants for field testing and it is important that a commercial view point is included in the research strategies from the start.

8.2 Large Scale Protocols

The large scale propagation of any crop requires a well defined protocol in which the main variables have been determined so that basic assumptions can be made for the design of an efficient production line. The protocol must not only include all the technical specifications, such as

concentrations of growth regulators etc. but must also take into consideration aspects such as:

- Timing
- Inputs
- Availability of space
- Costs
- etc.

The main variables that should be considered are:

- Average propagation efficiency of the tissues at all stages
- Average duration of each stage
- Average efficiency of the technicians
- Numbers of plants to be produced per month or week
- Number of working hours required for each part of the different processes
- Total number of working hours required per day or week
- Number of hours of air flow cabinet spaces
- Litres of culture media required per day or week
- Total number of culture dishes in the growth rooms at a single time over the whole production period
- Number of multiplication transfers for a clonal line
- Costs of reagents and accessories

8.3 Design of a Large Scale Production Line

This is the most important, but also the most difficult task in efficiently running large scale production laboratories. The reason for this is that it entails many parameters that can continuously vary. The production line must therefore be a relatively simple and flexible system that can be re-programmed as many times as necessary. To learn to design and use a production flow scheme, it is advisable to do it initially by hand and to experiment with it while production is still limited to a small scale. Later on, the use of a computer programme is recommended to help to run the lab in a more efficient manner.

The total (accumulative) number/quantity of every variable must be calculated throughout the whole process to ensure that none of them is greater than the production capacity of the laboratory at any single moment. Particular attention should be paid to the capacity measurement of:

- Air flow cabinet space
- Growth room incubation capacity
- Sterilization volumes
- Number of culture dishes available

- Number of hours worked by technical staff per week or day
- *Ex-vitro* planting and incubation capacities

The production of a number of plants over a fixed period of time can be programmed in many different ways to accommodate all the above. It might imply increasing the number of clones that are propagated, reducing the number of multiplication transfers and the total final number of plants produced for a specific clone, or, alternatively, increasing the number of working hours over a certain period, etc. Tables 8.1a and b show how the production will vary according to the initial number of plants and the multiplication rate.

The running of a large micropropagation laboratory implies the planning of production of many different species and varieties each one of them, probably, with different culture requirements, propagation efficiencies etc. that must be produced according to demand.

The production strategy will not be the same if 50,000 plants have to be delivered altogether or over a period of two months, it will also vary according to whether the final product consists of unrooted micro cuttings or potted plants.

A production flow chart that takes all of the above into consideration must be drawn up to programme the production of each line. This chart should be adjusted as often as necessary until it matches the laboratory's proven capacity.

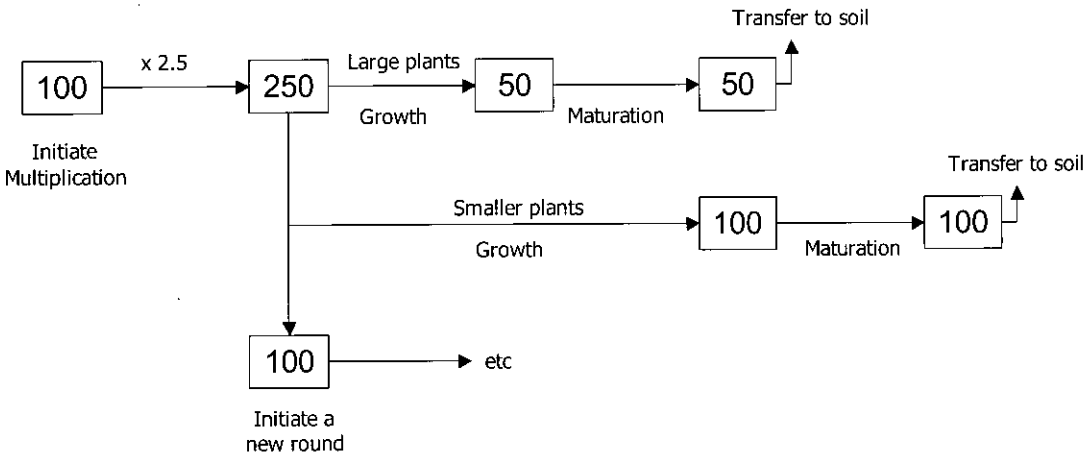
8.4 Flow Chart for Production Control (an example)

To illustrate the handling of the problems discussed above, a theoretical model flow chart for a large scale propagation of agaves is presented below. It is designed for the production of a constant number of plants over a period of 34 weeks starting with the planting of a 100 plants every day. For the sake of space and simplicity, the time scale in the flow chart is represented in weeks but in practice, the same scheme starts every day of the week for as many weeks as desired.

Under this scheme, biomass is built up and then maintained constant (in accordance with the production targets and the capacity of the laboratory).

This means that, in each new run, the same number of plants is transferred for multiplication so that the total biomass (Figure 8.1, curve B) and the number of manipulations required at any stage of the process do not increase and surpass the operational capacity of the technician or of the laboratory, as could happen if the biomass were increased without control (Figure 8.1 Curve A). The basic scheme is the following:

100 plants, at an assumed multiplication rate of 2.5X, produce 250 new plants in four weeks (depending on the multiplication rate, the numbers could be 300 or 400).



Independently of the numbers, only one hundred of these plants are replanted in multiplication media to start a new run, while the rest are transferred to growth media.

According to their size, the plants are kept growing for 4 or 8 additional weeks before they are transferred to pre-adaptation/rooting conditions for another 4 weeks, after which they are transplanted to soil. Each run (in this example) will therefore produce 150 new plants, while maintaining a biomass of 100.

This scheme is initiated and repeated as many times as necessary to reach the desired level of production while maintaining a constant multiplying biomass (Figure 8.1 curve B). This should avoid bottle necks when production increases and there is a need to carry out several different types of transfers on a single day.

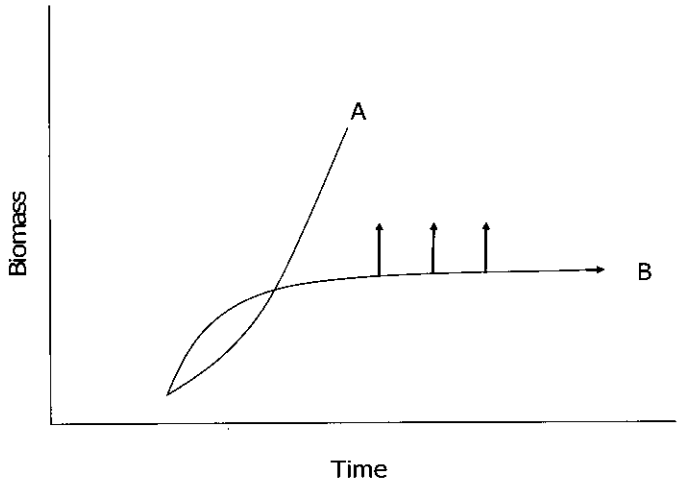


Figure 8.1 Biomass built

The flow chart is only an exercise to exemplify how to program and calculate the amount of work that will be required to propagate a specific species or variety. The real figures and times should be the actual data obtained from the experiments carried out with the species or variety in question, on the type of dish employed, the manual ability of the operator and the delivery schedule. Contamination or any other type of expected losses must be taken into account in these calculations.

A different flow chart must be drawn for each species or variety according to its own propagation characteristics, and each operator must have his/her own chart to program his/her own work. The lab manager must combine all the charts to program the laboratory's output.

If production of more than one species/variety is proceeding simultaneously, or if their production figures differ, a chart must be produced for each one of them.

It is convenient to program transfers in constant periods of time, preferably weeks, in order to have the same number of operations every day of the week and make all the different runs coincide. In other words, multiplication transfer will be done in three, four or five weeks but not in periods of six or twelve days. In this way, the weekends will not cause irregularities in the program.

8.5 How to Use the Flow Chart

By adding the different operations performed each day (green and yellow coloured boxes) it will be possible for the operator to calculate the maximum work he/she will have to carry out on a single day at the peak of production. These numbers must not exceed his/her capability to do the job in a specified number of hours. The numbers in red are the plants leaving the *in vitro* culture that are being transplanted to soil.

At the bottom of the table are the total numbers of plants being transplanted to soil and transferred to other *in vitro* stages, as well as the number of culture dishes manipulated daily that, in turn, depends on the type of culture dish used and the number of plants cultivated per dish. Here we are considering Magenta boxes with a minimum of 20 plants each.

It can be observed that the numbers increase during the build up stage and then become constant for a period of time, the length of which depends on the total number of plants which are being produced or the dates programmed for delivery etc. Finally, the numbers decrease until the last plants leave the laboratory. The use of this type of chart also allows the lab manager to compare the ability of each operator.

8.5.1 How Many Plants Can be Produced?

According to the flow chart, building up the biomass for the proposed scheme will take 16 weeks. During the first 12 there will be no output of plants and from week 13 to 16 only 50 plants a day will be produced. From week 17 onwards, 150 plants will come out of the *in vitro* system every day.

Considering the biomass build up period (weeks 1-16) and extending the production flow for 16 more weeks for a total of 50 weeks (1 year), the total production would be 27,500 plants /year.

A whole year (50 weeks) in full production (150 plants/day) would generate 37,500 plants/year.

At the peak of the production line only 20 magenta boxes are handled per day. Considering that one technician should be capable of handling at least 7 culture boxes every hour and approximately 30 Magenta boxes in a 4 h shift, the expected production for each technician in a 4 h shift would be 37,500 X 1.5 = 56,250 plants per year. Even allowing for 10% losses in the process and rounding up figures, the scheme presented should produce: 50,000 plants/4 hour shift/year

One laminar air flow cabinet could be used for three 4 hour shifts per day so that 150,000 plants per year could be produced in each one of them. The assumption is that to produce one and a half million plants per year a minimum of ten airflow cabinets and 30 shifts of 4 hours will be required. This calculation is only for *in vitro* production; washing and planting have not been taken into account.

8.5.2 Calculation of Required Inputs

Other factors that ought to be taken into account to ensure that there are no shortages of space or materials include the following:

8.5.2.1 *Culture Media*

Each of the 30 magenta boxes that is required every day per 4 hour shift will be filled with 50 ml of culture media for a total of 1.5 L / day, 7.5 L/week and a total of 375 L / year.

The stocks of salts and chemicals required to prepare 1,000 litres are presented in Annex I.

8.5.2.2 *Culture Dishes*

Assuming a transfer cycle of four weeks, around 750 boxes will be incubated in the growth rooms at a single time at the peak of the production line.

The magenta boxes will be in a cycle of washing, refilling, sterilization, planting and incubation implying a minimum of 1,250 magenta boxes in the production line.

8.5.2.3 *Incubation Capacity*

A standard 1.20mX 0.60-m. shelf in a growth room holds approximately 120 Magenta boxes, implying that 6 to 7 linear metres of shelves will be filled at all times with 750 boxes.

8.5.2.4 *Calculation of Production Costs*

It is difficult to calculate the costs of a specific micropropagation procedure. However, a rough preliminary estimation can be made on the basis of the efficiency calculated so far for the different varieties. This will make it possible to calculate how many plants can be produced in the laboratory in one year with the resources (space, manpower etc.) available and this number should be divided by the total expenses incurred (which have been previously discussed) that include :

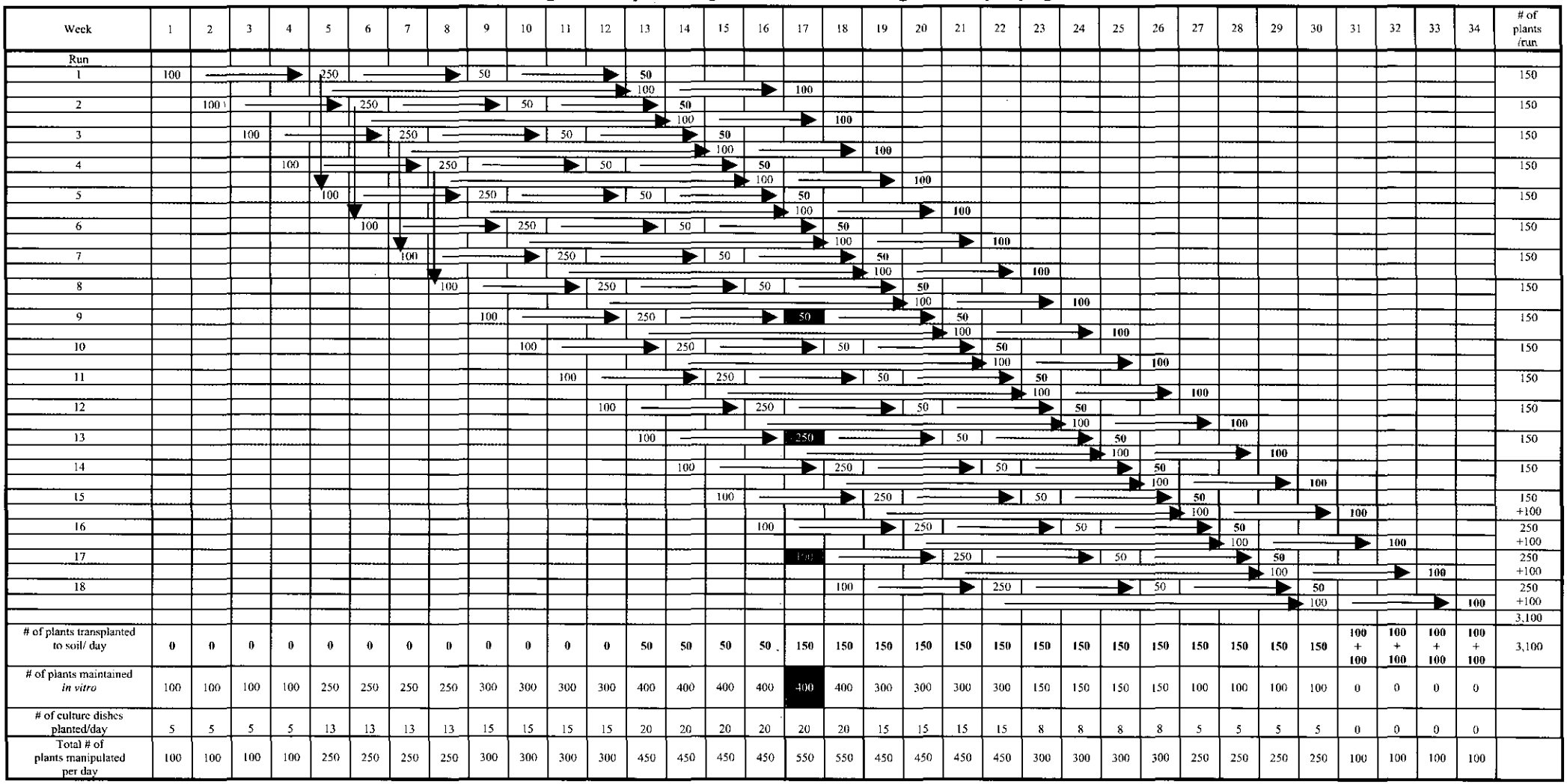
- Full salaries of all the personnel involved.
- Total energy consumption.
- Total number of liters of culture media employed multiplied by the cost of one liter of culture media (Annex I)
- Total yearly expenses on all other accessories and consumables.

The cost of one litre of culture media is calculated by adding the proportional cost of the amount of each substance used.

This calculation does not include the total investment in construction and equipment which must be calculated independently according to the depreciation rates for each component in each country.

MODEL FLOW CHART

For large scale planning and control of large scale propagation



Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	# of plants /run
Run																																			
1	100				250				50				50																						150
													100				100																		
2		100				250				50				50																					150
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3			100				250				50				50																				150
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# of plants transplanted to soil/ day	0	0	0	0	0	0	0	0	0	0	0	0	50	50	50	50	150	150	150	150	150	150	150	150	150	150	150	150	150	150	100 + 100	100 + 100	100 + 100	100 + 100	3,100
# of plants maintained *in vitro*	100	100	100	100	250	250	250	250	300	300	300	300	400	400	400	400	400	400	300	300	300	300	150	150	150	150	100	100	100	100	0	0	0	0	
# of culture dishes planted/day	5	5	5	5	13	13	13	13	15	15	15	15	20	20	20	20	20	20	15	15	15	15	8	8	8	8	5	5	5	5	0	0	0	0	
Total # of plants manipulated per day	100	100	100	100	250	250	250	250	300	300	300	300	450	450	450	450	550	550	450	450	450	450	300	300	300	300	250	250	250	250	100	100	100	100	

Table 8.1a *In vitro* production of plants starting with a biomass of 300 plants and assuming various multiplication efficiencies

Multiplication efficiency	Number of transplants					
	1	2	3	4	5	6
1.5 X	300	450	675	1,012	1,518	2,278
2.0X	300	600	1,200	2,400	4,800	9,600
2.5X	300	750	1,875	4.687	11,718	29,295
3.0X	300	900	2,700	8,100	24,300	72,900
3.5X	300	1,050	3,675	12,862	45,018	157,565
4.0X	300	1,200	4,800	19,200	76,800	307,200

Table 8.1b *In vitro* production of plants starting with a biomass of 2,000 plants and assuming various multiplication efficiencies

Multiplication efficiency	Number of transplants					
	1	2	3	4	5	6
1.5 X	2,000	3,000	4,500	6,750	10,125	15,187
2.0X	2,000	4,000	8,000	16,000	32,000	64,000
2.5X	2,000	5,000	12,500	31,250	78,125	195,312
3.0X	2,000	6,000	18,000	54,000	162,000	324,000
3.5X	2,000	7,000	24,500	85,750	300,125	1,050,437
4.0X	2,000	8,000	32,000	128,000	512,000	2,084,000

Chapter 9

Alternative New Techniques for the Micropropagation of Agaves

Chapter 9

Alternative New Techniques for the Micropropagation of Agaves

9.1. Introduction

As we have seen in chapters 3 and 4, most micropropagation processes are carried out through direct organogenesis in vessels with a culture medium solidified with a gelling agent to create a substrate on which the plants can grow and multiply. In spite of its general use, this method has some disadvantages:

- The culture conditions are not homogeneous since not all the tissues are in contact with the nutrient medium
- The plants need to be continuously transferred to new containers with different fresh medium and growth regulator concentrations
- The multiplication efficiencies can be very low, making it necessary to carry out many transfers in order to increase the biomass

In consequence, micropropagation is a long and labour intensive method that greatly increases the production costs of the plants produced *in vitro* and is only economically viable on a commercial scale in the case of high value added species (Chu, 1995). Manual labour represents the main expense in commercial micropropagation; although estimates vary from place to place it accounts for a minimum of 60-70% of the overall production costs.

In order to simplify the whole process, reduce production costs and make micropropagation available to a larger number of species it is necessary to develop simpler, more efficient and cheaper methods that can produce higher numbers of plants, faster and with less labour.

For this purpose two different, but complementary strategies offer the most promising outcome:

- Somatic embryogenesis
- Culture in liquid media (Temporary immersion bioreactors)

9.2 Somatic Embryogenesis

The induction of somatic embryos from undifferentiated tisúes (calli) is a very efficient multiplication method that has been successfully applied to several commercial species (Bajaj, 1995).

9.2.1 Somatic Embryogenesis in *Agave fourcroydes*

In spite of this potential, the micropropagation of *Agave* tissues through somatic embryogenesis is still a field being investigated.

In order to generate thousands of plants in a single step, the method required is indirect somatic embryogenesis (see chapter 3) for which it is necessary to induce and culture sufficient embryogenic calli, in a reproducible manner, that will produce high numbers of embryos that will mature and germinate in a more or less synchronized form.

Although there are publications about the induction of somatic embryos in *Agave* species (see chapter 3), the processes reported out have not been very efficient and only a few embryos are produced over periods of several months (Piven et al., 2003).

For this technique to be viable as a micropropagation method it needs to be fast and fully reproducible and the genetic stability of the plants produced has to be demonstrated.

9.2.2 The RITA System

This situation could, nevertheless, change rapidly through the use of temporary immersion bioreactors that have been used with great success in the production of somatic embryos from several important plantation crops that include rubber, coffee etc.(Teisson C. & Alvard D., 1995).

Recent results such as the one shown in Figure 9.1 (Robert et al., unpublished results) have demonstrated that the induction of somatic embryos from already induced embryogenic calli was improved by 2000% when the calli were cultured in a RITA temporary immersion bioreactor (Figure 9.1) and the analysis of the genetic stability of these plants through AFLP showed them to be as stable as the plants produced through direct organogenesis (Reyes, Ph. D. thesis).

Although these results are very promising, much work will still be required before these methods can be regularly used for micropropagation.

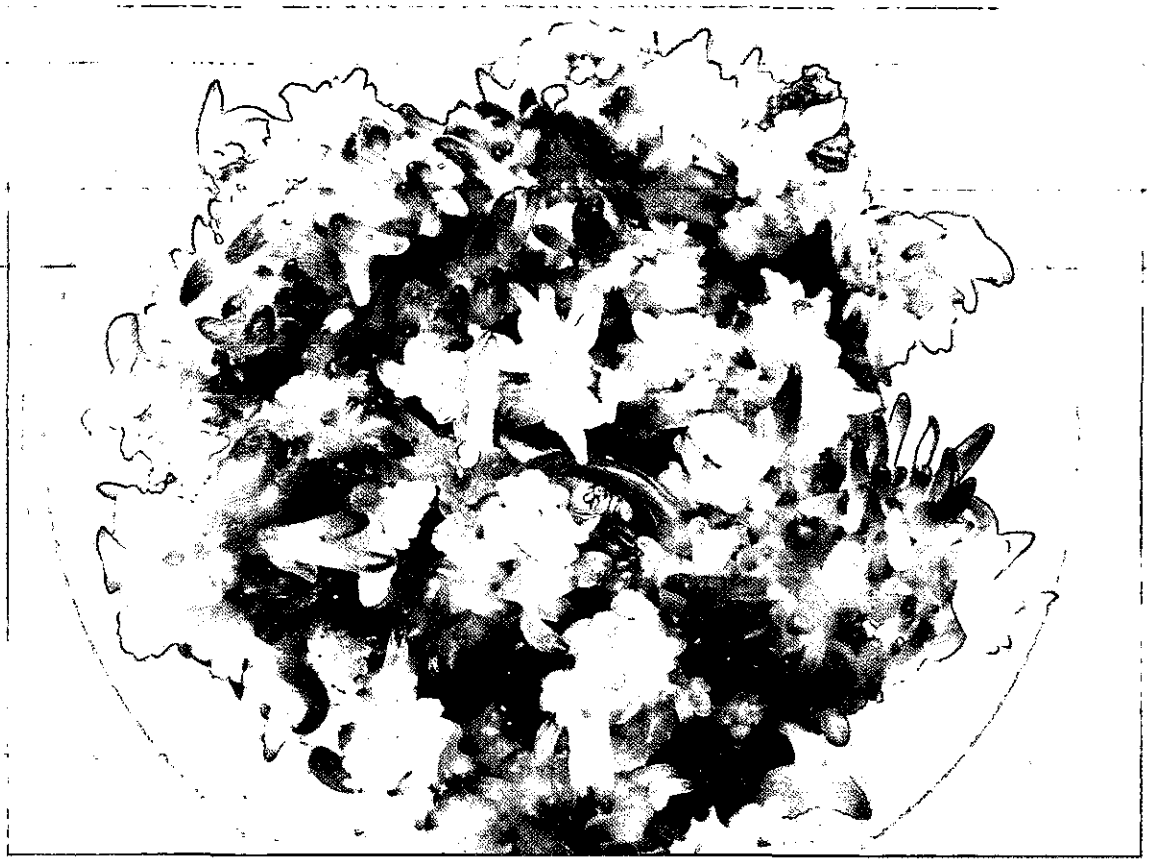

Figure 9.1 *Agave* somatic embryogenesis in temporary immersion bioreactors (RITA).

9.3 Culture in Liquid Media

Culture in liquid media, in theory, offers the best conditions for efficient, large scale micropropagation since it is more amenable to automation thereby reducing manual labour (Debergh, 1988; Aitken-Christie, 1991, Ziv et al., 1998).

Its many advantages include:

- Being able to change the culture media, instead of moving the plants one by one from one container to the next.
- Not requiring jellifying substances that considerably increase the costs
- Providing a direct and more homogeneous source of nutrients for the cultured tissues
- The possibility of sterilization through micro filtration.

Culture in liquid medium, however, also has some disadvantages:

- The plants become vitrified
- Microbial contamination is more difficult to control and

- It is more complicated to scale up because of the size of the plantlets being propagated.

9.3.1 Temporary Immersion

A method that combines the advantages of both semi-solid and liquid culture media is the Temporary Immersion System designed by Teisson and collaborators (1995). This system alternates short periods of total immersion in liquid medium with longer ones of complete aeration by displacing the culture media, by means of pressurized air, from a lower chamber to an upper one that holds the plant tissues (Figure 9.2).

Satisfactory results for the propagation of various species have been reported using two bioreactors based on this principle (Teisson et al., 1995; Etienne et al., 1999; Escalona et al., 1999 and Espinosa et al., 2002).

The RITA system has proved to be very useful for the culture of somatic embryos, but this system is not adequate for the culture and propagation of larger plants such as agaves (Figure 9.2). The RITA bioreactor is too small for the propagation of large vitroplants and the sponge that supports the plants at the base of the top chamber maintains very high humidity that induces a certain degree of vitrification in the plants at the bottom.

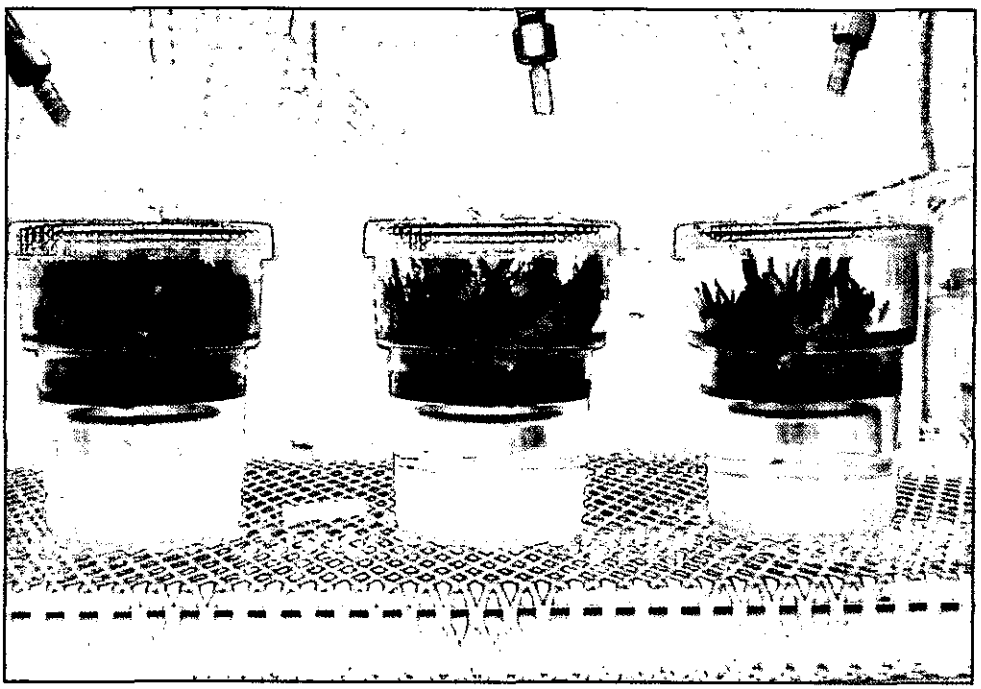

Figure 9.2 Multiplication stage in temporary immersion bioreactors (RITA).

A larger bioreactor, such as the one used by Escalona and collaborators (2003) is too big and is therefore difficult to sterilize. Small and big bioreactors require complicated systems of tubes and filters connecting

them to the machines that drive the liquid medium from one vessel into another which are not easy to manipulate.

9.4 BioMINT

In order to eliminate these difficulties, a middle sized, modular bioreactor (BioMINT) (Registered trade mark, PA/a2004/003837) was designed (Robert et al., 2004c).

The movement of the liquid medium from one vessel to the other is passively driven by gravity and therefore does not require being connected to pumps or air compressors. It has a larger volume capacity (1200 ml.) compared to the RITA (250ml.), but it is small and light enough to be easily autoclaved and transported from the airflow cabinet to the culture room. Its structural simplicity permits easy opening and closing that expedites the introduction and removal of the cultured plant materials and the change of the liquid culture media according to the objectives and needs of the micropropagation system.

BioMINT can be used for all stages of the micropropagation process, from induction to *in vitro* weaning, of many species. It considerably reduces the amount of labour required for transfers and the period of time required for some micropropagation stages, and it produces larger and more vigorous plants compared to the ones cultured in semi-solid medium in magenta boxes.

Its low production costs and efficacy make it an economic alternative for low value added species such as agaves.

9.4.1 The Equipment

The BioMINT unit consists of two cylindrical vessels (made of polycarbonate and fully autoclavable) that are closed at one end. The two vessels are joined together through their open ends by an adaptor piece (Figure 9.3a). One vessel is for the plant tissues and the other for the liquid culture medium. The adaptor piece is closed in the middle by a perforated plate that permits the free flow of the liquid culture medium while keeping the plants or tissues in their place when the bioreactors change position.

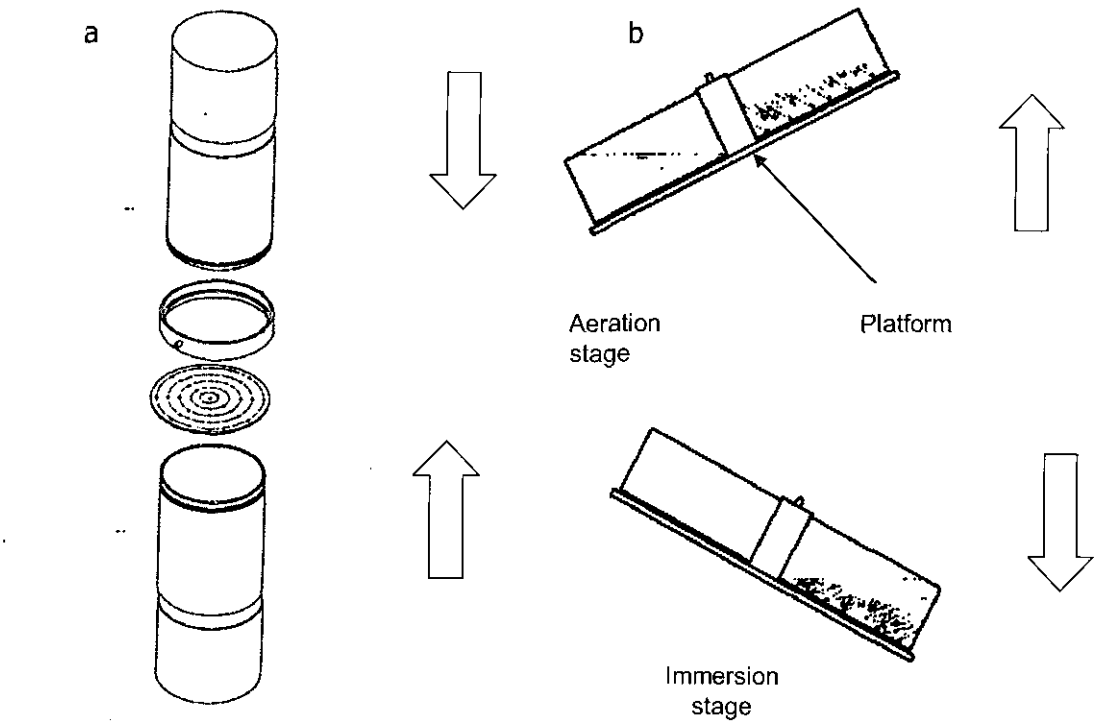

Figure 9.3 BioMINT: Modular temporary immersion bioreactors.

The liquid culture media is displaced from one vessel to the other by gravity when the bioreactors change their inclination (Fig 9.3b) by means of the SyB (see-saw (PA/a2004/003837)) electromechanical unit that consists of a set of four platforms (Figure 9.4) on which the bioreactors are placed that alternate positions, moving up and down driven by a piston connected to an electrical motor. The whole system is automatically controlled by a programmable control panel that regulates the timing and the speed with which the platforms change position. The system of 4 platforms and 36 BioMINT units is equivalent to a 45 litre bioreactor.

If the culture medium needs to be changed, the BioMINT can be taken back to the air flow cabinet and opened by removing the vessel containing the plant tissues and coupling it to another one containing fresh or a different medium before returning it to the SyB.

Microbial contamination is a more serious problem in liquid culture than in semi-solid culture, because the bacteria or fungi from one contaminated plant will rapidly spread to all the others. However, contamination is more easily controlled in the modular bioreactors than in large ones because there is a smaller number of plants exposed to the contaminated one which reduces the risk, and also because the modular design allows for easy individual operation and treatment of the vessels with small amounts of antimicrobial chemicals (See chapter 7).

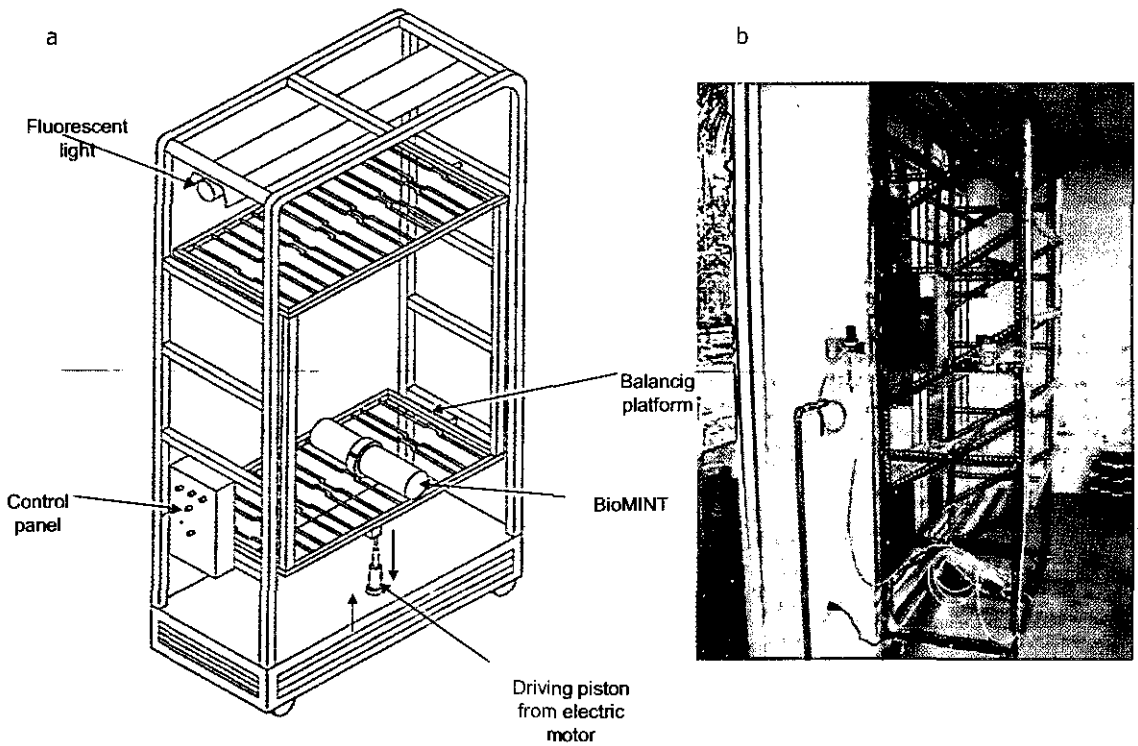

Figure 9.4 Diagram and prototype of the SyB module to operate the BioMINTs.

The use of BioMINT prototypes (Figure 9.5a) has produced excellent preliminary results for the propagation of *Agave fourcroydes* Lem. Immersion/aeration cycles as short as 5-10 minutes immersion for every 2 to 4 hours of aeration are sufficient to produce very vigorous plants that grow faster than the ones in magenta boxes (Figure 9.5b). Because of the predominantly aerated conditions in which they are grown, the plants adapt very well to transplant to soil conditions.

The most important advantage of this system is that the manipulations that have to be performed to propagate this species have been reduced to less than 40%.

The results obtained so far suggest that BioMINT might become the standard procedure for the micropropagation of *Agave* species in the near future.

Figure 9.5 Propagation of *Agave fourcroydes* in a temporary immersion prototype.

Unidad de Biotecnología, Centro de Investigación Científica de Yucatán A.C. México

Final Considerations

Final Considerations

Problems and the Need for Innovation

Until very recently, the cultivation of most industrial *Agaves* was based on old practices with almost no technological innovation. Stagnant or falling demand and low prices did not seem to justify any significant investment in scientific research into these rather underappreciated plants. However, the recent appearance of a number of weaknesses in agave-related industries has begun to highlight the role that research and technology can play in transforming them into world class businesses.

Faced with a sharp increase in demand for Agave-based alcoholic beverages, the tequila industry found itself unable to respond with sufficient speed because of the difficulty of increasing the availability of planting material in a short lapse of time. At the same time, a fungal disease began to sweep through the plantations, causing huge losses and an upward spiral of prices. Only then did the tequila industry begin to turn to science for answers and new solutions.

In Kenya and Tanzania growers have repeatedly said that the present productivity of H 11648 does not resemble that of the original hybrid. This is hardly surprising if today's plantations are the result of sixty years of vegetative propagation through bulbils with little or no field selection by the growers and no scientific genetic improvement programme.

As more research is carried out, more is known about the present and potential utility of these plants and there is a growing sense of what scientific innovation can achieve to make them more efficient at satisfying human needs.

The Contribution of Micropropagation

Genetic improvement is one of the two basic strategies for increasing the yields of crops. Most economically important species are continuously bred to develop varieties that are resistant to disease or that can cope with different environmental conditions. However, agaves are difficult to breed because of their long life cycle and because some of them do not reproduce sexually.

Plant tissue culture can contribute to breeding programmes through the rapid propagation of new genotypes that, with traditional techniques, would take many years to introduce. Therefore this technique is particularly useful with species that present long life spans and/or reproduce vegetatively, such as agaves.

It can also contribute to the generation of new genetic variability through the induction, selection and propagation of natural or induced mutation. Two other applications of tissue culture are: the production of healthy materials for the control of disease dispersal and the conservation of endangered species.

Disease dispersal is a particularly serious problem in today's world of open global markets and special attention must be given to the way in which micropropagated plants are used and distributed. The conservation of germplasm is an issue that urgently requires more attention and tissue culture offers two alternatives: the rescue of endangered species through micropropagation and the long term storage of germplasm.

Tissue Culture for Developing Countries

Compared with genetic engineering, which, in addition to being costly, also lacks social acceptance in some countries because of its potential environmental hazards, tissue culture is a relatively simple and inexpensive methodology, well within the reach of most countries. Even those countries whose human and financial resources are very limited can obtain practical results in a few years with a relatively small investment in scientific training, infrastructure, equipment and reagents. In fact, tissue culture represents one of the best techniques for developing countries in their search for solutions to agronomic problems and crops, particularly those that are ignored by the large companies because of the low financial rewards they stand to get (Eastmond and Robert, 1990; 1992). Consideration of the establishment of a tissue culture laboratory should therefore be assigned a high priority by governments and industries concerned about improving local crop production.

In spite of being a relatively cheap technique, however, this does not imply that tissue culture can be operated successfully in makeshift facilities. It still requires a proper laboratory with adequate equipment and a basic level of training, if good results are to be obtained. Growers all over the world are usually more concerned with reducing production costs and increasing their profit margins in the shortest possible time, than in making sure that their procedures are adequate and safe. This lack of concern can lead to a decrease in yields or, on occasions, be the prelude to a failure to detect an epidemic before it affects a whole industry.

As a result of the fungal epidemic that caused so much damage to the tequila industry, most large tequila companies have now changed their attitudes to short term "cost-saving" at the expense of preventive measures. Many of the most important companies are now investing heavily in contracted research with scientific institutions, and in

micropropagation and some are even establishing their own research and micropropagation facilities.

Other agaves, which are exploited on a smaller scale, require direct government support and/or research through public institutions to modernize the industries. For example, the government of the State of Sonora is funding the technification of bacanora (*Agave angustifolia* Haw) production as part of its regional development strategy (Nuñez, 2004) which includes the establishment of nurseries of micropropagated materials. The relevance that biotechnology has for *Agave* cultivation in Mexico was recently manifested in the number of presentations on this topic during the meeting on "the agaves of economic importance in Mexico (IV International symposium on agavaceae and Nolinaceae)" that took place in the city of Mérida last March.

The CFC program has managed to provide the sisal industry in East Africa with the capability of exploiting biotechnology for its own benefit. Now it is up to the laboratories in Kenya and Tanzania to interact with the growers to design a genetic improvement strategy to increase yields and produce healthier plants. As demand for higher quality plants increases, commercial micropropagation laboratories will find they can play a role with the large scale production of the successful materials.

Integration

Throughout this document it has been argued that *in vitro* tissue culture is essential for the modernization of the cultivation of agaves and that micropropagation offers immediate applications towards this aim. But, the question remains of how to access the technology. The strategy that many of the largest transnational corporations currently follow is "vertical integration". This means acquiring the in-house capability for the whole productive chain, from basic research to commercialization. In spite of the advantage of total control over production and marketing, it is, however, an expensive strategy which only the giant corporations can afford. The alternative strategy for producers with smaller budgets, academic and commercial research laboratories, and government agencies is "horizontal integration". This involves forming strategic alliances with similarly positioned institutions from different sectors, offering different skills and capabilities, the sum of which make up the whole productive chain. In the case of agave-derived products, for example, the final commercializing business could link up with a research institution for specialized knowledge input and a commercial micropropagation company to carry out the large scale production. The exact modernization strategy adopted by a particular industry will, of course, depend on its specific objectives, resources and time frame.

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Glossary of Terms Used in this Manual

Glossary of terms use in this manual

Abscisic acid. A phytohormone implicated in the control of many plant responses to abiotic stress, such as extent of stomatal opening under water deficit (i.e. drought) conditions.

Adventitious. Arising in an irregular or unusual position such as roots arising other than from the radicle or primary root system; adventitious buds forming along a stem, generally in response to an injury. Not in the usual place.

Agar. A polysaccharide solidifying agent used in nutrient media preparations and obtained from certain types of red algae (Rhodophyta). Both the type of agar and its concentration can affect the growth and appearance of cultured explants.

Air flow cabinet. Laminar Flow Cabinets are self-contained workstation units available in either horizontal or vertical laminar flow models. A laminar flow cabinet creates a particle-free working environment by taking air through a filtration system and exhausting it across a work surface in a laminar or unidirectional air stream. Commonly, the filtration system compromises of a pre-filter and a HEPA filter. Because the air within the cabinet does not contain any airborne particles, it is also sterile. The laminar flow cabinet is enclosed on the sides and kept under constant positive pressure in order to prevent the infiltration of contaminated room air.

Aneuploid. An organism or cell having a chromosome number that is not an exact multiple of the monoploid (x) with one chromosome being present in greater (e.g., trisomic 2n + 1) or lesser (e.g., monosomic 2n - 1) number than the normal diploid number.

Apical meristem. The un-differentiated tissue at the tip of a shoot or root, that by division produces the precursors of the differentiated tissues, including vegetative, reproductive or floral organs.

Aseptic. Sterile, free of contaminating organisms (bacteria, fungi, algae but not generally including viruses, and particularly not internal symbionts).

Asexual. Without the involvement of fertilisation, propagation by division or the production of bulbils, offsets or stolons.

Autoclave. Apparatus in which media, glassware, etc. are sterilized by high temperature and pressure.

Auxin. A group of plant growth regulators (natural or synthetic) which stimulate cell division, enlargement, apical dominance, root initiation, and flowering.

Axillary bud. A bud formed in an axil.

Axillary meristem. A meristem found at the axil of a leaf (synonymous with lateral meristem).

Bagasse. The dry dusty pulp that remains after juice is extracted from agave or similar plants.

Bioreactor. A tank in which cells, cell extracts or enzymes carry out a biological reaction. Often refers to a fermentation vessel for cells or micro-organisms.

Browning. Discoloration of freshly cut surfaces of plant tissue due to phenolic oxidation. In plant tissue culture, it may indicate a nutritional or pathogenic problem, generally leading to necrosis.

Bud. Buds are compressed shoots. In normal development, buds form at certain growth sites or "nodes" on the surface of a stem during the growth of that stem. That pattern usually determines the growth pattern of the plant for the rest of its life. are formed by meristematic tissue which has differentiated into stem, leaf and floral parts. In normal development buds form at certain growth sites or nodes on a stem during the growth of that stem. Buds can be divided into two classes, fruiting and vegetative. Vegetative buds will grow into branches, fruiting buds will form flowers and then fruit. Vegetative buds cannot be distinguished from fruiting buds in all species of tree, as some buds are combined.

Bulbil. A small vegetatively derived plant produced on an inflorescence.

Callus. (pl.: calli) A protective tissue, consisting of parenchyma cells, that develops over a cut or damaged plant surface. 2. Mass of undifferentiated, thin-walled parenchyma cells induced by hormone treatment. 3. Actively dividing non-organized masses of undifferentiated and differentiated cells often developing from injury (wounding) or in tissue culture in the presence of growth regulators.

CAM metabolism. Crassulacean Acid Metabolism, a metabolic adaptation allowing temporary storage of carbon-dioxide as C-4 organic acids (e.g. malate, aspartate) and later release of carbon-dioxide, by decarboxylation of these acids, for fixation into sugars. This allows uptake of carbon-dioxide during the cool nights, when water loss by transpiration is relatively low, while providing a store of carbon-dioxide for photosynthesis during the day. C-4 metabolism is typical of Agaves, Cactaceae, Crassulaceae and tropical grasses and can support high rates of growth under optimum conditions.

Clone. A plant that has been propagated asexually and therefore has attributes that are identical in every respect to the parent. Daylilies do not revert to a different form, nor do they produce sports, naturally

occurring variants that have distinctly new or different characteristics (a quite common event with hosta).

Clonal propagation. Asexual propagation of many new plants (ramets) from an individual (ortet); all have the same genotype.

Cloned strain or line. A strain or line descended directly from a clone.

Cytokinins. Plant growth regulators characterized as substances that induce cell division and cell differentiation. In tissue culture, these substances are associated with enhanced callus and shoot development. The compounds are derivatives of adenine.

Cultivar. A plant originated or selected artificially. Names of cultivars may be appended after the species name, (e.g. *Lithops bromfieldii* "Sulphurea") sometimes following the letters "cv."

Culture medium. A nutrient system for artificially growing bacteria or other cells.

Culture room. A dedicated room for maintaining cultures, often in a controlled environment.

Cuticular waxes. Cuticular waxes are complex mixtures consisting of a variety of components. Certain substance classes or single constituents may dominate the mixture and contribute up to 80 and 70 percent of the total, respectively. The aliphatic components have long, unbranched carbon chains of 16 to 36 carbon atoms (in the case of alkyl esters, from C34 to > C60) and carry one or two functional groups. Most of the cyclic components are pentacyclic triterpenoids. These substance types have been found in most of the waxes investigated so far and may even dominate wax composition. Recently, an additional and theretofore unknown wax fraction consisting of oligomeric polar (aliphatic and cyclic) components has been isolated and characterized

Differentiation. The progressive physiological and morphological changes in cells, tissues, organs or the whole plant during development from meristem tissue to a mature or adult form. Differentiated tissues are usually more functionally specialised than undifferentiated ones.

Direct embryogenesis. The formation in culture, on the surface of zygotic or somatic embryos or on explant tissues (leaf section, root tip, etc.), of embryoids without an intervening callus phase. Opposite: indirect embryogenesis.

Direct organogenesis. Formation of organs directly on the surface of cultured intact explants. The process does not involve callus formation. Opposite: indirect organogenesis.

Donor plant. Original plant of seedling origin from wich members of a clone have originated.

Dormancy. A period in the life of an animal (hibernation and aestivation) or plant during which growth slows or completely ceases. Evolved to allow survival of adverse environmental conditions. Annual plants survive the winter as dormant seeds, while many perennial plants survive as dormant tubers, rhizomes, or bulbs. Premature breaking of seed dormancy post harvest can be a major problem for maintaining nutritional and/or functional quality, while difficulties in breaking dormancy will lead to poor germination of the crop. See: quiescent.

Elite line. Used to describe a highly improved, well adapted breeding line or variety with superior.

Elite plant. A phenotypically superior plant in a plant breeding programme.

Embryo. The new plant developing from the fertilised egg cell (zygote) in the fertilised seed.

Embryogenesis. *In vitro* formation of plants from plant tissues, through a pathway closely resembling normal embryogeny from the zygote. Somatic cell embryogenesis is an alternative technique. The generation of embryos has two stages: initiation and maturation. Initiation needs a high level of the group of plant hormones called auxins; maturation needs a lower level.

Endangered species. A plant or animal species in immediate danger of extinction because its population numbers have reached a critical level or its habitats have been drastically reduced.

Explant. A portion of a plant aseptically excised and prepared for culture in a nutrient medium.

Ex vitro. Organisms removed from tissue culture and transplanted; generally plants to soil or potting mixture.

GelriteTM. The brand name of a Pseudomonas-derived refined polysaccharide used as a gelling agent and agar substitute.

Genotype. The genetic constitution of an organism.

Genus. Grouping of plants below family and above species that unites a group of species with distinctive characteristics in common. It is the first name in Latin

Germplasm. The sum total of all hereditary material in a single (interbreeding) species.

Gibberellin. A class of plant growth regulators which are active in the elongation, enhancement of flower, fruit and leaf size, germination, verbalization and other physiological processes.

Growth regulator. A synthetic or natural compound that at low concentrations elicits and controls growth responses in a manner similar to hormones.

Haploid. Having a single set of chromosomes.

Hectare (ha). A metric unit of area equal to 100 Ares (2.471 acres) and equivalent to 10,000 square meters (107,639 square feet).

Hybrid. Progeny from an inter-specific (or inter-generic) fertilisation, often of intermediate appearance to the parents.

Hyperhydricity. Physiological abnormality occuring in tissue culture; formerly the word vitrification was used

Inflorescence. A flower cluster, including bracts, on a stalk.

In vitro. Outside the organism, or in an artificial environment. Applied for example to cells, tissues or organs cultured in glass or plastic containers.

***In vitro* rooting.** Shoots are treated with auxin, usually either by putting the shoots on auxin-containing medium, or by dipping them in auxin solution and planting on hormone-free medium.

In vivo. The natural conditions in which organisms reside. Refers to biological processes that take place within a living organism or cell under normal conditions.

Induction of shoots. Stable cultures are multiplied. The cytokinin in the medium promotes bud growth and several shoots grow for every one placed in culture. Every four to eight weeks, the shoots are divided and placed on fresh medium to repeat the process. Eventually, the surplus shoots are channeled into propagation.

Indirect embryogenesis. Plant embryo formation on callus tissues derived from explants, including zygotic or somatic embryos and seedlings. Opposite: direct embryogenesis

Indirect organogénesis. Plant organ formation on callus tissues derived from explants. Opposite: direct organogenesis

Juvenile. Young or immature, not having flowered.

Leaf primordium. A lateral outgrowth from the apical meristem, which will become a leaf when fully developed and expanded.

Magenta box. A type of plastic container frequently used for plant micropropagation and tissue culture.

Meristem. Undifferentiated but determined plant tissue, in which the cells are capable of active division and differentiation into specialized tissues such as shoots and roots.

Meristem culture. A tissue culture containing meristematic dome tissue without adjacent leaf primordia or stem tissue. The term may also imply the culture of meristemoidal regions of plants, or meristematic growth in culture.

Micropropagation. Miniaturized in vitro multiplication and/or regeneration of plant material under aseptic and controlled environmental conditions.

Molarity. The number of moles of a substance contained in a kilogram of solution.

Monocotyledon. A large group of flowering plants with a single cotyledon that initially emerges from the seed. The Agavaceae and Liliaceae are monocotyledons.

Morphogenesis. The development, through growth and differentiation, of form and structure in an organism.

Mutant. An organism or an allele bearing a mutation. Usually applied when a characteristic change in phenotype can be recognized.

Mutation. A sudden, heritable change appearing in an individual as the result of a change in the structure of a gene (= gene mutation); changes in the structure of chromosomes (= chromosome mutation); or in the number of chromosomes (= genome mutation). cf genetic diversity; genetic drift.

Mycorrhiza. Fungi that form an association with, or have a symbiotic relationship with roots of more developed plants.

Organogenesis. The initiation of adventitious or de novo shoots or roots from callus, meristem or suspension cultures. See micropropagation; regeneration.

Pasteurized. Having been subjected to pasteurization in order to halt fermentation.

Pathogen. A disease-causing organism (generally microbial: bacteria, fungi, viruses; but can extend to other organisms: e.g. nematodes etc.). Synonym: infectious agent. See: latent agent.

Peat or Peat Moss. Decayed remains of ancient plants, added to soil to increase the soil's ability to absorb and hold moisture.

Phenolics. Compounds with hydroxyl group(s) attached to the benzene ring, forming esters, ethers and salts. Phenolic substances produced from newly explanted tissues are liable to oxidise, and as a result form coloured compounds visible in nutrient media.

Photoperiod. The length of daylight or period of daily illumination provided for growth.

Photosynthesis. A chemical process by which green plants synthesize organic compounds from carbon dioxide and water in the presence of sunlight.

Phytopathogen. A plant pathogen.

Plantlet. A small rooted shoot developed from seed or from cultured cells either by embryogenesis or organogénesis.

Ploidy number. Number of sets of chromosomes present in a living organism. A diploid organism has two sets of chromosomes, a triploid three sets and so on in tetraploids, pentaploids, hexaploids etc.

Polyploid. Tissue or cells with more than two complete sets of chromosomes, that results from chromosome replication without nuclear division or from union of gametes with different number of chromosome sets, hence triploid (3x), tetraploid (4x), pentaploid (5x), hexaploid (6x), heptaploid (7x), octoploid (8x)).

Pre-adaptation. A step in *in vitro* propagation of plants characterised by the establishment of an aseptic culture.

Pre-conditioning. A step in *in vitro* propagation of plants, aiming to prepare the plant material for successful introduction in tissue culture.

Protocol. The step-by-step experiments proposed to describe or solve a scientific problem, or the defined steps of a specific procedure.

Rhizogenesis. De novo formation of roots.

Rhizome. An underground stem from which roots, aerial stems or leaves can arise directly.

Rooting. Culture of isolated root tips of apical or lateral origin to produce in vitro root systems with indeterminate growth habits.

Saprophyte. An organism (generally a fungus) that depends on dead plant or animal tissue for its source of nutrition and metabolic energy.

Seasonal variation. Seasonal variation represents the effect of climatic and institutional events that repeat more or less regularly each year.

Seedling. Any recently-sprouted plant.

Semisolid culture. Partly solid media culture; having a rigidity and viscosity intermediate between a solid and a liquid; "a semisolid mixture".

Sexual reproduction. The process where two cells (gametes) fuse to form one fertilized cell or zygote. cf asexual reproduction; gamete; hybrid.

Shoot tip. The terminal bud (0.1 - 1.0 mm) of a plant, which consists of the apical meristem (0.05 - 0.1 mm) and the immediately surrounding leaf primordia and developing leaves, and adjacent stem tissue. Synonym: shoot apex.

Somaclonal variation. Phenotypic evidence of mutation arising from cell or tissue culture.

Somatic. Referring to vegetative or non-sexual stages of a life-cycle.

Somatic embryogenesis. The process of differentiation of somatic embryos either from explant cells (direct embryogenesis), or from callus generated from explants (indirect embryogenesis). Synonym: asexual embryogenesis.

Species. A class of potentially interbreeding individuals that are reproductively isolated from other such groups having many characteristics in common. A somewhat arbitrary and sometimes blurred classification; but still quite useful in many situations.

Stoma (pl.: stomata). A pore in the epidermis of the leaf or stem of a plant, which allows the exchange of gases, including water vapour, to and from the intercellular spaces. Sometimes used loosely to refer to the pore along with its associated pair of guard cells. Synonym: stomate. See: stomatal complex.

Substrate. 1. A compound that is altered by an enzyme. 2. Food source for growing cells or micro-organisms. 3. Material on which a sedentary organism lives and grows.

Suckers. Shoots or small plants arising from the base of a larger plant.

Tissue Culture. The *in vitro* culture of cells, tissues or organs in a nutrient medium under sterile conditions.

Transgenic. Possessing genes from a different organism

Variety. A naturally occurring subdivision of a species, with distinct morphological characters and given a Latin name according to the rules of the International Code of Nomenclature. A taxonomic variety is known by the first validly published name applied to it so that nomenclature tends to be stable.

Vegetative. Growth of leaves and stems as opposed to flowers. A method of non-sexual reproduction by means of offsets, cuttings.

Vitrification. Formation of amorphous ice during the process of cryopreservation; formerly also used to describe the process of hyperhydricity.

Vitroplants. Vitroplants are obtained by *in vitro* micropropagation of mother plants.

Water stress. Occurs when plants are unable to absorb enough water to replace that lost by transpiration. Short-term water stress leads to turgor loss (wilting). Prolonged stress leads to cessation of growth, and eventually plant death

Annexes

Annex I

Preparation of Plant Tissue Culture Media

Preparation of Stocks

To prepare tissue culture media it is necessary to prepare concentrated stock solutions of their components. The stock solutions not only facilitate the preparation of large amounts of culture media since reagents only have to be weighed once and can be stored in small volumes, but make sure that all the salts have dissolved completely. The different solutions are mixtures of salts that do not precipitate each other at the concentrations they are present in the stocks.

The stock solutions are prepared weighing the right amount of substance and dissolving it in half the final volume with continuous stirring in a large beaker at room temperature. Once the salts are completely dissolved the final volume is adjusted using a volumetric flask. The stock solutions are stored in dark (amber) flasks in a refrigerator.

Stock Solutions of Growth Regulators

The right amount of the growth regulator is dissolved in 1/10 of the final volume with continuous stirring. If the free acid form of the growth regulator is used, an equimolar amount of NaOH can be added to facilitate its dissolution. Once the substance is completely dissolved, the final volume is adjusted using a volumetric flask and stored as above.

Mixing the Stock Solutions

For the preparation of, for example, 1l of MSB medium, a large beaker is filled with 500ml of distilled water. With continuous stirring, add 30 g of sucrose, 10 ml of each of the 6 stocks, in the same order of their numbers (see Annex 1), 10ml of growth regulators stocks. Once all the components have been mixed the final volume is adjusted using a volumetric flask.

The solution is returned to a large beaker and the pH meter electrode is immersed in the solution. To adjust the pH to 5.75, either 1mM HCl or 1mM NaOH is added, drop by drop, while stirring gently.

Finally, the required amount of gelling agent (agar, gelrite or phytagel) is added. While stirring to produce a homogenous mixture, a fixed volume of the latter is dispensed into the culture dishes, e.g. Magenta boxes are filled with 50 ml each.

The filled culture dishes are sterilized in a pressure cooker or an autoclave at 121°C at a pressure of 1.1 kg/cm^2 for 20 minutes. To avoid irregular gelling, the culture dishes should only be removed while they are still hot or once they have cooled completely.

The culture dishes can be stored in a cold room at 4°C but should be incubated at 27°C for a couple of days before use in order to detect and eliminate any contaminated ones.

Calculation of Cost of 1000 litres of MSB-10 Culture Medium

No.	Chemical	Size (g)	Sigma Catalogue Number	Price USD	Amount (mg) required/liter	Amount (g) required for 10 l of stock	Cost/USD
1	Glycine	100	G - 7126	19.3	2.00	2.0	0.39
	Nicotinic acid	100	N - 4126	28.8	0.50	0.5	0.14
	Pyridoxine	25	P 8666	22.5	0.50	0.5	0.45
	Thyamine	100	T - 4625	42.7	0.10	0.1	0.04
	Innositol	100	I - 5125	35.8	100.00	100.0	35.80
2	KI	100	P 2963	54.7	0.83	0.8	0.45
	$Mn SO_4 . H_2O$	500	M - 7899	54	16.90	16.9	1.83
	$H_3 BO_3$	500	B - 9645	30.3	6.20	6.2	0.38
	$Zn SO_4 . 7H_2O$	100	Z - 0251	22.1	8.60	8.6	1.90
	$Na_2 MoO_4 . 2 H_2O$	100	M - 1651	23.9	0.25	0.3	0.06
	$Cu SO_4 . 5H_2O$ *	250	C - 2857	56.6	0.025	0.0	0.01
	$Co Cl_2 . 6H_2O$ *	25	C - 8661	20.3	0.025	0.0	0.02
3	$Mg SO_4 . 7H_2O$	500	M - 2643	85.3	370.00	370.0	63.12
	$K H_2 PO_4$	500	P - 5379	39.9	170.00	170.0	13.57
4	KNO_3	500	P - 7391	16.9	1820.00	1820.0	61.52
	$NH_4 NO_3$	500	A - 3745	34.9	400.00	400.0	27.92
5	$Ca Cl_2 . 2H_2O$	500	C - 5080	50	440.00	440.0	44.00
6	$Fe SO_4 . 7H_2O$	500	F - 8263	49.73	27.80	27.8	2.76
	Na_2 EDTA $2H_2O$	500	EDTA	50.7	37.30	37.3	3.78
	Sub- total						**258.14**
	6 BAP	25	B - 3408	324.8	10.0	10.00	129.92
	2,4-D	100	D - 7299	17.8	0.025	0.03	0.004
	Distilled water (liter)	20		2.68	1.00	1000.00	0.134
	Sucrose grade II	25000	S - 5391	231.7	30000.00	30000.00	278.040
	Gelrite	5000	G - 1910	1016.4	1750.0	1750.0	355.740
	Agar	25000	A - 1296	2140	1750.0	1750.0	149.800
	Sub- total						**913.64**
	Total						**1171.77**
	Cost of one litre of media						**1.2**

Salt Composition of most Common Plant Tissue Culture Media

Murashige and Skoog (MS) Medium

No. Stock	Compound	mg/l	Molarity	Stock conc. mg/l	ml of stock/1
	Sucrose	30000.0	87.64 mM		30.0 g
1	Glycine	2.0	26.64 μM	20	10
	Nicotinic acid	0.5	4.06 μM	0.5	
	Pyridoxine	0.5	2.43 μM	0.5	
	Thiamine	0.1	0.296 μM	1.0	
	Inositol	100.0	555.0 μM	1000.0	
2	KI	0.83	5.0 μM	0.083	10
	$Mn\ SO_4 . H_2O$	16.90	100.0 μM	1.69	
	$H_3\ BO_3$	6.20	100.3 μM	0.62	
	$Zn\ SO_4 . 7H_2O$	8.60	29.0 μM	0.86	
	$Na_2\ MoO_4 . 2\ H_2O$	0.25	1.03 μM	0.025	
	$Cu\ SO_4 . 5H_2O$	0.025	0.10 μM	*	
	$Co\ Cl_2 . 6H_2O$	0.025	0.105 μM	*	
3	$Mg\ SO_4 . 7H_2O$	370.0	1.5 mM	37.0	10
	$K\ H_2\ PO_4$	170.0	1.25 mM	17.0	
4	KNO_3	1900.0	18.8 mM	190.0	10
	$NH_4\ NO_3$	1650.0	20.6 mM	165.0	
5	$Ca\ Cl_2 . 2H_2O$	440.0	2.99 mM	44.0	10
6	$Fe\ SO_4 . 7H_2O$	27.8	100.0 μM	2.78	10
	$Na_2\ EDTA\ 2H_2O$	37.3	100.2 μM	3.73	

Schenk and Hildebrandt (SH) Medium

No. Stock	Compound	mg/l	Molarity	Stock conc. mg/l	ml of stock/1
	Sucrose	30000.0	87.64 mM		30.0 g
1	Nicotinic acid	5.0	40.6 μM	0.5	10
	Pyridoxine	0.5	2.43 μM	0.05	
	Thiamine	5.0	14.82 μM	0.5	
	Inositol	1000.0	5.55 mM	100	
2	KI	1.0	6.024 μM	0.1	10
	$Mn\ SO_4 . H_2O$	10.0	59.0 μM	1.0	
	$H_3\ BO_3$	5.0	80.8 μM	0.5	
	$Zn\ SO_4 . 7H_2O$	1.0	3.47 μM	0.1	
	$Na_2\ MoO_4 . 2\ H_2O$	0.1	0.413 μM	0.01	
	$Cu\ SO_4 . 5H_2O$	0.2	0.80 μM	*	
	$Co\ Cl_2 . 6H_2O$	0.1	0.42 μM	*	
3	$Mg\ SO_4 . 7H_2O$	400.0	1.62 mM	40.0	10
4	KNO_3	2500.0	24.7 mM	250.0	10
	$NH_4\ H_2\ PO_4$	300.0	2.608 mM	30.0	
5	$Ca\ Cl_2 . 2H_2O$	200.0	1.36 mM	20.0	10
6	$Fe\ SO_4 . 7H_2O$	15.0	53.9 μM	1.5	10
	$Na_2\ EDTA\ 2H_2O$	20.0	53.7 μM	2.0	

Gamborg (B5) Medium

No. Stock	Compound	mg/l	Molarity	Stock conc. mg/l	ml of stock/1
	Sucrose	20000.0	58.43 mM		20.0 g
1	Nicotinic acid	1.0	8.12 μM	0.1	10
	Pyridoxine	1.0	4.86 μM	0.1	
	Thiamine	10.0	29.64 μM	1.0	
	Inositol	100.0	555.0 μM	10.0	
2	KI	0.75	4.52 μM	0.075	10
	$Mn\ SO_4 . H_2O$	10.0	59.16 μM	1.0	
	$H_3\ BO_3$	3.0	48.52 μM	0.3	
	$Zn\ SO_4 . 7H_2O$	2.0	6.95 μM	0.02	
	$Na_2\ MoO_4 . 2\ H_2O$	0.25	1.03 μM	0.0125	
	$Cu\ SO_4 . 5H_2O$ *	0.025	0.1 μM	*	
	$Co\ Cl_2 . 6H_2O$ *	0.025	0.11 μM	*	
	$NaH_2\ PO_4 . H_2O$	150.0	1.087 mM	15.0	
3	$Mg\ SO_4 . 7H_2O$	250.0	1.014 mM	50.0	5
4	KNO_3	2500.0	24.7 mM	125.0	20
	$(NH_4)_2\ SO_4$	134.0	1.014 mM	6.7	
5	$Ca\ Cl_2 . 2H_2O$	150.0	1.02 mM	15.0	10
6	$Fe\ SO_4 . 7H_2O$	27.8	100.0 μM	2.78	10
	$Na_2\ EDTA\ 2H_2O$	37.3	100.2 μM	3.73	

Withe Medium

No. Stock	Compound	mg/l	Molarity	Stock conc. mg/l	ml of stock/1
	Sucrose	20000.0	58.43 mM		20.0 g
1	Glycine	3.0	39.9 mM	0.3	10
	Nicotinic acid	0.5	4.06 μM	0.05	
	Pyridoxine	0.1	0.48 μM	0.01	
	Thiamine	0.1	0.296 μM	0.01	
2	KI	0.75	4.51 μM	0.075	10
	$Mn\ SO_4 . H_2O$	5.3	31.36 μM	0.53	
	$H_3\ BO_3$	1.5	24.26 μM	0.15	
	$Zn\ SO_4 . 7H_2O$	3.0	10.43 μM	0.3	
	$Na\ H_2PO_4 . 2\ H_2O$	16.5	119.57 μM	1.65	
3	$Mg\ SO_4 . 7H_2O$	720.0	2.92 mM	72.0	10
4	KNO_3	80.0	791.21 mM	8.0	10
	$Ca\ (NO_3)_2 . 4H_2O$	300.0	1.27 mM	30.0	
5	$Na_2\ SO_4$	200.0	1.408 μM	20.0	10
	KCl	65.0	871.78 μM	6.5	
6	$Fe\ (SO_4)_3$	2.5	6.25 μM	0.25	10

* 1 ml from a ministock of 2.5 mg/ml

Annex II

Reagents and Equipement

List of Equipment

- PH Meters (See page 1156-1159, Cat. Cole-Parmer 2004-05).
- Pipettors (See page 1222, Cat. Cole-Parmer 2004-05).
- Stirring hot plates (See page 2457-2460, Cat. SIGMA 2004-05).
- Analytical balances (See page 70-72, Cat. Cole-Parmer 2004-05).
- Portable balance (See page 48-60, Cat. Cole-Parmer 2004-05).
- Peristaltic pump (See page 1290-1353, Cat. Cole-Parmer 2004-05).
- Sterilizers (See page 1718-1720, Cat. Cole-Parmer 2004-05).
- Laminar flow cabinet (See page 634, Cat. Cole-Parmer 2003-04).
- Sterillizer, dry bead (See page 2279, Cat. SIGMA 2004-05).
- Bunsen burners (See page 135, Cat. Cole-Parmer 2004-05).
- Togs and tweezers (See page 884, Cat. Cole-Parmer 2003-04).
- Spatulas (See page 870, Cat. Cole-Parmer 2003-04).
- Scalpel handles (See page 770, Cat. Cole-Parmer 2003-04).
- Vacuum desiccators PYREX® (See page 796, Cat. Cole-Parmer 2003-04).
- Service cart (See page 770, Cat. Cole-Parmer 2003-04).
- Orbital shakers (See page 1674-1677, Cat. Cole-Parmer 2003-04).
-

List of Glasware

- Beakers NALGENE (See page 710, Cat. Cole-Parmer 2003-04).
- Beakers PYREX® (See page 714, Cat. Cole-Parmer 2003-04).
- Cylinders PYREX® (See page 792, Cat. Cole-Parmer 2003-04).
- Cylinders NALGENE (See page 794, Cat. Cole-Parmer 2003-04).
- Glass serological pipettes PYREX® (See page 1210, Cat. Cole-Parmer 2003-04).
- Bottles PYREX® (See page 726, Cat. Cole-Parmer 2003-04).
- Bottles NALGENE (See page 730, Cat. Cole-Parmer 2003-04).
- Carboys NALGENE (See page 762, Cat. Cole-Parmer 2003-04).
- Wash bottles NALGENE (See page 743, Cat. Cole-Parmer 2003-04).
- Petri dishes PYREX® (See page 798, Cat. Cole-Parmer 2003-04).

- Sample vials (See page 894, Cat. Cole-Parmer 2003-04).
- Büchner funeles ceramic (See page 819, Cat. Cole-Parmer 2003-04).
- Funeles PYREX® (See page 824, Cat. Cole-Parmer 2003-04).
- Magenta® vessels (See page 2291, Cat. SIGMA 2004-05).
- Culture tubes PYREX® (See page 888, Cat. Cole-Parmer 2003-04).
- Vessels for plant tissue culture (baby food jars) (See page 2293, Cat. SIGMA 2004-05).
- Stir bars (See page 1729, Cat. Cole-Parmer 2003-04).
- Weighing boats (See page 2133, Cat. SIGMA 2004-05).
- Tape (See page 882, Cat. Cole-Parmer 2003-04).
- Lab markers (See page 852, Cat. Cole-Parmer 2003-04).

List of Reagents

*Auxins***

- 4-Chlorophenoxyacetic acid (Cat. No. C 0413).
- 2,4-Dichlorophenoxyacetic acid (Cat. No. D 7299).
- (2,4-Dichlorophenoxy) acetic acid sodium salt monohydrate (Cat. No. D 6679).**
- 3-Indolacetic acid (Cat. No. I 2886).
- Indole-3-acetic sodium salt (Cat. No. I 5148).
- Indole-3-acetyl-L-aspartic acid (Cat. No. I 9387).
- Indole-3-butyric acid (Cat. No. I 5386).
- Indole-3-butyric acid potassium salt (Cat. No. I 7512).
- Indole-6-propionic acid (Cat. No. I 8639).
- Methyl indole-3-acetate (Cat. No. I 9770).
- 1-Naphthaleneacetic acid (Cat. No. N 0640).
- 1- Naphthaleneacetic acid solution (Cat. No. N 1641).
- 1- Naphthaleneacetic acid potassium salt (Cat. No. N 1145).
- 2-Naphthoxyacetic acid (Cat. No. N 3019).
- Phenylacetic acid (Cat. No. P 6061).
- Picloram (Cat. No. P 5575).
- 2,4,5-Trichlorophenoxyacetic acid (Cat. No. T 5785).
- 2,3,5-triiodobenzoic acid (Cat. No. T 5910).

*Cytokinins***

- Adenine (Cat. No. A 5665).

- Adenine hemisulfate salt (Cat. No. A 2545).
- 6-Benzylaminopurine (Cat. No. B 3408).
- 6-Benzylaminopurine hydrochloride (Cat. No. B 5920).
- N,N'-Diphenylurea (Cat. No. D 7535).
- Kinetin (Cat. No. K 0753).
- Kinetin solution (Cat. No. K 3253).
- Kinetin hydrochloride (Cat. No. K 1885).
- Thidiazuron (Cat. No. P 6186).
- Zeatin (Cat. No. Z 0164).
- Trans-zeatin (Cat. No. Z 0876)
- Trans-zeatin hydrochloride (Cat. No. Z 2753).
- Trans-zeatin-riboside (Cat. No. Z 3541).

Gelificantes

- Agar plant cell culture tested (Cat. No. A 4550).**
- Agargel plant cell culture tested (Cat. No. A 3301).**
- Gel rite gellan gum (Cat. No. G 1910).**
- Phytagel agar substitute gelling agent (Cat. No. P 8169).**
- Oromex and Agrosym

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  Fax: +1 (202) 202 822-6410.
  Dr. Assaf Guri. Tel: 1 (856) 541-1141.
  http://www.ppm4plant-tc.com/contact_information.htm

*Cole Parmer catalog

** Sigma-Aldrich catalog

Recommended agrochemicals for the preventive control of plagues and diseases during the acclimatization stage.

Technical name	Common name	Presentation	Composition	Manufacturer	Use	Mode of Application
Metan-potassium	Busan 69GE	Aqueous solution	N-Potassium methyl dithiocarbamate	Buckman Laboratories Inc.	Soil Fumigant	Irrigation 100 cc/lt water/m^2
Captan	Captan	Wettable powder	N (trichloromethyltio) ciclohex-4-ene-1,2 dicarboximide	Drexel Chemical Company Inc	Fungicide	Foliar spray 2 - 5 g/lt water
Chlorothalonil	Bravo	Liquid	2,4,5,6-tetrachloroisophthalonitrile	Syngenta	Fungicide	Foliar spray 1 - 3 cc/lt water
Agrimicin	Agrimicin 500	Wettable powder	Streptomicin sulphate Oxitetraciclin clorhydrate Tribasic copper sulphate	Pfizer	Bactericide	Foliar spray 1-2 g/lt water
Methomyl	Lannate	Soluble powder	S-methyl-N-(methylcarbamoyloxi) thioacetimidate	Dupont	Insecticide	Foliar spray 5 - 6 g/10 lt water
Methamidophos	Monitor	Liquid	O,s-dimetil phosphoramidothioate	Bayer	Insecticide	Foliar spray 1 - 2 cc/lt water

Annex III

Laboratories that Identify Microbial Contaminants

Biosan Laboratories, Inc.
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Phone (586) 755-8970 / (800) 253-6800
Fax (586) 755-8978 e-mail lesley@biosan.com

Gibson Laboratories, Inc.
1040 Manchester Street • Lexington, KY 40508
(800) 477-4763 • (859) 254-9500 • Fax: (859) 253-1476
E-Mail: gibsonlabs@alltel.net

BBC LABORATORIES, INC.
1217 N. Stadem Drive, Tempe, AZ 85281
Phone (480)967-5931 Fax (480)967-5036

Korea Microbiological Laboratories, Ltd.
1Ra-107, Shihwa industrial complex, Jeongwang-dong Siheung-si Gyeonggi-do 429-450 Korea. phone 82 - 31 – 4986104, 82 - 31 – 4986107, Home page www.komilab.co.kr. Contac Yang, Yongjin.

BIOHIDRICA®
Biotecnologias del Agua Ltda. Anibal Aracena 571 (Nunoa)
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biohidrica@biohidrica.cl

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Urdiñola Norte. 360. CP 25000. Saltillo, Coahuila,. México.
Tel. 844 4123756. Fax. 844 4123 755.
Cisef@mcsa.net.mx.

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Annex IV

Directory of Laboratories and Scientists Working on Agave Tissue Culture

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Printed in Austria
V.04-58084—October 2004—1,000

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