In vitro mutation breeding of bananas and plantains

Final reports of an FAO/IAEA co-ordinated research programme organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture from 1988 to 1993

INTERNATIONAL ATOMIC ENERGY AGENCY

June 1995
The IAEA does not normally maintain stocks of reports in this series. However, microfiche copies of these reports can be obtained from

INIS Clearinghouse
International Atomic Energy Agency
Wagramerstrasse 5
P.O. Box 100
A-1400 Vienna, Austria

Orders should be accompanied by prepayment of Austrian Schillings 100,— in the form of a cheque or in the form of IAEA microfiche service coupons which may be ordered separately from the INIS Clearinghouse.
IN VITRO MUTATION BREEDING OF BANANAS AND PLANTAINS
IAEA, VIENNA, 1995
IAEA-TECDOC-800
ISSN 1011–4289
© IAEA, 1995
Printed by the IAEA in Austria
June 1995
Mutation breeding has been successfully applied to cereals, legumes and other seed-propagated crops, in which the induced mutants segregate as homozygotes. In vegetatively propagated crops, many important mutant cultivars have been released, not only ornamental plants but also major fruit tree crops. The major objective of previous studies has been the development of cultivars with increased resistance or tolerance to biotic or abiotic stresses, e.g. disease or lodging resistance, quality improvement for higher market values, early or late maturity for better agriculture practices, etc.

There is an urgent need for the application of mutation breeding techniques in vegetatively propagated crops such as the bananas. In most of the northern developed countries, banana is known primarily as a dessert fruit. However, bananas and plantains (cooking bananas) are major sources of starch in tropical countries, and are also important as export items. Currently, yields of *Musa* crops are declining as plantings are attacked by various disease organisms, e.g. black sigatoka in Latin America, *Fusarium* wilt in Southeast Asia, and banana bunchy-top virus (BBTV) disease in Pakistan.

Unfortunately, improvement of bananas and plantains by conventional breeding techniques is difficult due to problems associated with sterility. Even if a promising disease resistance gene were found in a related species, it would be very difficult to incorporate it into superior cultivars. In these instances mutation breeding may be a practical method for the improvement of these crops. Recent development of in vitro culture techniques makes the application of mutation breeding possible with bananas and plantains. Large numbers of somatic embryos, or somatic tissues, can be irradiated in a small container. Thus, the treatment of the large populations normally required for mutation breeding is possible.

A co-ordinated research programme (CRP) was organized to study the feasibility of developing mutation breeding protocols in *Musa*, and in solving the problems likely to be encountered in the implementation of such an approach. This TECDOC contains the final reports of the participants of the CRP. The report from the late Dr. F. Novak and his group, that was presented at the Second Research Co-ordination Meeting (RCM) held in San Jose, Costa Rica is also included. Dr. Novak who passed away on 19 July 1993, was a pioneer and a driving force behind the banana research programs at IAEA’s Seibersdorf Laboratory and elsewhere.

This document was jointly prepared by the Scientific Secretary, E. Amano, and by R.L. Jarret, United States Department of Agriculture’s Agricultural Research Service, whose contribution is acknowledged.
EDITORIAL NOTE

In preparing this publication for press, staff of the IAEA have made up the pages from the original manuscripts as submitted by the authors. The views expressed do not necessarily reflect those of the governments of the nominating Member States or of the nominating organizations.

Throughout the text names of Member States are retained as they were when the text was compiled.

The use of particular designations of countries or territories does not imply any judgement by the publisher, the IAEA, as to the legal status of such countries or territories, of their authorities and institutions or of the delimitation of their boundaries.

The mention of names of specific companies or products (whether or not indicated as registered) does not imply any intention to infringe proprietary rights, nor should it be construed as an endorsement or recommendation on the part of the IAEA.

The authors are responsible for having obtained the necessary permission for the IAEA to reproduce, translate or use material from sources already protected by copyrights.
CONTENTS

INTRODUCTION ................................................................. 7

Improvement of bananas (Musa cvs.) through in vitro anther culture ............ 9
M. Perea-Dallos

Genetic improvement of bananas through in vitro mutation breeding in Costa Rica .. 13
A.T. Valerin, R. Salazar, W. Navarro

Radiation mutation induction in 'China Tianbao' banana .......................... 17
P. Yang, C. Li, F. Wei, W. Huang, J. Zheng, D. Huang, R. He, J. Guo,
X. Huang, B. Chen, W. Lin

Improvement of banana (Musa cvs.) through in vitro culture techniques and induced mutations ............................................ 25
S.H. Siddiqui, A. Khatri, I.A. Khan, G. Nizamani, R. Khan

Effect of toxic filtrate of Fusarium oxysporum f.sp. cubense on the development of banana (Musa cvs.) shoot tips ........................... 31
B.M.J. Mendes, B.I.P. Rodrigues, A. Tulmann Neto

In vitro mutation breeding for the development of bananas with resistance to race 4, fusarium wilt (Fusarium oxysporum f. sp. cubense) ......................... 37

Somatic embryogenesis in bananas and plantains (Musa clones and species) ...... 45
A.D. Krikorian

Improvement of Musa through biotechnology and mutation breeding ............ 57

Study on mutation breeding of banana, 'Kluai Khai' ................................ 65
B. Silayoi, K. Wanichkul, S. Keawsompong, P. Saraduldhat, N. Singhaburaudom

CONCLUSIONS AND RECOMMENDATIONS .................................... 79

PARTICIPANTS IN THE CO-ORDINATED RESEARCH PROGRAMME ............... 85
INTRODUCTION

Less than a decade ago fewer than a handful of scientists worldwide were involved in biotechnological research with bananas and plantains. This situation has changed dramatically in the last ten years. More than a dozen laboratories are now actively involved in tissue culture, molecular genetic, transformation and basic plant pathology research with *Musa*. For the most part these research efforts are conducted in support of, or in direct collaboration with, on-going plant breeding programmes.

Much of the current interest in *Musa* improvement can be attributed to three factors. These are: 1) an increasing awareness of the vulnerability of bananas and plantains to attack by various disease organisms and insect pests, 2) an acknowledgement of the difficulty of breeding these crops using conventional techniques, and 3) the recognition of the social and economic importance of these crops to many millions of people worldwide.

An impressive amount of progress has already been made in the development and application of biotechnological approaches for *Musa* improvement. A few examples include:

- tissue culture protocols for the *in vitro* propagation of *Musa* clones via organogenesis, and somatic embryogenesis have been reported and are being further refined.
- successful cryopreservation of *Musa* cell suspensions has been achieved.
- molecular genetic markers have been used to study evolutionary and taxonomic relationships of *Musa* species and for estimating genetic diversity in *Musa* germplasm collections.
- a genetic linkage map of *Musa*, using molecular markers, has been constructed.
- progress has been made in the application of genetic transformation technology using the biolistic process.
- conditions for mutation induction, utilizing *in vitro* culture techniques in combination with $^{60}$Co $\gamma$ rays or EMS, have been established, and irradiation-induced mutants are now under field evaluation.
- an *in vitro* *Musa* germplasm repository has been established.

Recent advances in gene isolation technology, YAC and microsatellite gene mapping, and transformation technologies suggest that the next ten years will be a very exciting period for *Musa* biotechnology researchers. In much the same way that tissue culture and mutation induction techniques have been melded into an effective system for *Musa* improvement, so will molecular genetic and transformation technologies merge. Ultimately all of these approaches will be used to support both conventional and mutation breeding programmes as *Musa* improvement efforts become increasingly multi-disciplinary.

The reports presented in this TECDOC represents the progress made to date in the application of mutation breeding and *in vitro* culture techniques for the production of disease resistant bananas and plantains. This work was inspired by, and largely directed by, the late Dr. F. J. Novak, in whose laboratory protocols for the regeneration of banana via somatic embryogenesis, and the irradiation of somatic embryos for mutation induction, were first developed. These significant advancements opened the way for the broader application of mutation induction and tissue culture techniques for the generation of disease resistant bananas and plantains.
IMPROVEMENT OF BANANAS (Musa cvs.) THROUGH IN VITRO ANther CULTURE

M. PEREA-DALLOS
Departamento de Biologia, Facultad de Ciencias,
Universidad Nacional de Colombia,
Bogota, Colombia

Abstract

Agricultural products play a great role in the Colombian economy, and the banana is one of the most important. Since 1981, one of the more serious problems effecting production of this crop is the fungus Mycosphaerella fijiensis sp. difformis, that causes black sigatoka disease. Most of the recent efforts to control this disease have been directed towards the identification of clones tolerant or resistant to this disease. One alternative approach is the use of anther culture to obtain resistant haploid plants. Diploid clones (Musa - AA) have been used as a model in this study. The results presented here identify the most appropriate stage of anther development for callus induction and proliferation, and treatments that reduce tissue browning.

1. INTRODUCTION

Bananas and plantains are two of the most important staple and cash crops for several million people in the developing countries [1]. Since the beginning of large-scale banana production, Colombia has been one of the most important banana and plantain export producers. Most plantain production occurs on farms that are located in the mountains, where coffee is the main crop. Under these conditions plantains are grown to furnish food for the coffee growers (as a "survival crop"). A "survival" crop implies security in the form of cheap food, not only for the farmers of the growing regions, but also for Colombia's various social groups.

The genetic improvement of bananas and plantains through conventional breeding methods has been hampered, due to the lack of understanding about the genetics of the plant, the pathogens attacking the crop, the interaction and co-evolution of host and pathogens, and the various factors effecting sterility that result in the production of non-viable seed [2]. Sterility in edible bananas is the result of a complex of factors that are directly related to the ploidy (triploidy) of the plant and the attendant meiotic abnormalities associated with triploidy. Fruit development is the result of parthenocarpy. These characteristics are genetically controlled and may have arisen through gene mutations in fertile diploids [3, 4, 5, 6, 7].

A combination of traditional cross-hybridization techniques and the use of biotechnology could provide a powerful tool for the development of new Musa clones with desirable features such as, for example, resistance or tolerance to diseases. Diploid clones of both wild (seed-fertile) and edible bananas are essential as starting material for banana breeding, as sources of genetic resistance or tolerance to the major banana diseases. Sources of resistance to many diseases and insects attacking Musa (except Banana Bunchy Top Virus), have been identified in the extensive collections of diploid Musa acuminata and M. balbisiana [8].

2. ANther CULTURE

The importance of anther culture to crop improvement has been discussed elsewhere. Significant advances in anther culture techniques have been made in the last two decades. Anther culture of monocotyledonous plants has been extensively researched over a number of years, since many important crop plants belong to this group. Problems associated with strong genotypic effects and high proportions of albino regenerants have seriously hindered the progress in this area [9].
A large number of plant species have been successfully regenerated from cultured anthers or pollen grains harvested at different states of development. However, in *Musa* clones, especially the widely cultivated clones, most of the genotypes do not produce viable pollen grains or egg cells. This phenomenon has prevented the use of many valuable clones in conventional hybridization improvement programs. Anther culture of more genotypes needs to be attempted in order to determine the interaction of genotype with media composition and environmental conditions. In view of the importance of haploids in plant breeding, it is highly desirable to investigate various factors concerning the effect of environment and media composition on the induction of cell division and differentiation. Anther culture is not currently used frequently in *Musa* breeding programs due to the strict dependence of plant regeneration on genotype. However, there are attempts to resolve this problem. Very recently, Bakry and Horry [10] regenerated plantlets from cultured banana anthers derived from AA diploids. The plantlets were diploids that had originated from unreduced microspores.

3. MATERIALS AND METHODS

3.1. Plant material

Experiments were carried out using three selected diploid clones of banana: Bocadillo, Pisang lilin, and Malascensis. These materials were collected from nursery fields at the Instituto Colombiano Agropecuario (ICA) at Carepa-Uraba. In keeping with the objectives of the study to determine the appropriate state of inflorescence development suitable for culture, inflorescences were chosen at different stages of development, for example when the bunch emerged 15 cm, and when the bunch had emerged 30 - 35 cm. This latter developmental stage corresponded to the time when the bunch began to bend downwards. Considering the asynchronous development of anthers in *Musa*, several hands were selected for use in the present studies. The most appropriate stage of the development was when the inflorescence had 1 - 12 hands.

Pollen grains were cultured when they were in the uni-nucleate stage and when the exine and intine layers were observable. The collected inflorescences were stored at 4 °C for 4 to 7 days.

3.2. Culture procedure and media

These materials were disinfected in 5 % (v/v) bleach (Clorox) solution for 45 minutes, followed by several rinses in sterile water. The isolation of anthers was carried out under aseptic conditions and they were placed onto Murashige and Skoog basal medium [11], supplemented with various concentrations of (3,6-dichloro-o-anisic acid (dicamba) at 5, 10, 15, 20, or 25 μM, 2 g/l casein hydrolysate and 60 g/l sucrose. The pH of the media was adjusted to 5.8. Four anthers were placed in each culture vessel and then incubated in the dark at 28 °C.

To avoid explant browning due to the phenolic compounds, several concentrations of activated charcoal (0.5, 1.0, and 2 %) and polyamines (spermine 5, 50 and 100 μM; spermidine 250 and 500 μM and, putrescine 250 and 500 μM) were tested. After the appearance of callus, explants were subcultured weekly.

4. RESULTS AND DISCUSSIONS

Callus was first observed 26 days after culture initiation. Callus development was observed in all anthers of the inflorescence.

Activated charcoal was not effective in reducing the browning of calli, but various concentrations of polyamines were very useful in reducing this browning. Intensive blackening was a serious problem during the establishment of anther cultures. However, the addition of polyamines to the medium helped to control this phenomenon and enhanced the callus induction frequency. As seen from the effect of polyamines on browning, it is important to include these compounds during culture initiation. Also, it is necessary to identify the optimal concentration.
Mengoli and Bagni [12] reported that the low conversion rate of grape somatic embryos into plantlets could be due to the very high polyamine content relative to the levels found in zygotic embryos.

**Table I.** Percent of anthers forming callus when cultured in the presence of dicamba

<table>
<thead>
<tr>
<th>Concentration of Dicamba (μM)</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bocadillo</td>
</tr>
<tr>
<td>5</td>
<td>88</td>
</tr>
<tr>
<td>10</td>
<td>75</td>
</tr>
<tr>
<td>15</td>
<td>49</td>
</tr>
<tr>
<td>20</td>
<td>32</td>
</tr>
<tr>
<td>25</td>
<td>15</td>
</tr>
</tbody>
</table>

*Fig.1. Effect of dicamba on the growth of callus from anthers of diploid bananas.*
Acknowledgements

The author is indebted to the Joint Program IAEA/FAO for support of part of this work, and also to Dr. R. Bayona, Director of Laboratorio de CENIBANANO and Dr. M. Mayorga, Director of Regional ICA at Carepa-Uraba who provided the plant materials. The author also acknowledges AUGURA and the Universidad Nacional for their support of this research. Gratitude is expressed to M. Espitia for his help in the typing of the manuscript.

REFERENCES


GENETIC IMPROVEMENT OF BANANAS THROUGH IN VITRO MUTATION BREEDING IN COSTA RICA

A. T. VALERIN, R. SALAZAR, W. NAVARRO
Plant Genetics Program,
Universidad Nacional,
Heredia, Costa Rica

Abstract

Fifty corms of the banana cultivar Gran Enano (AAA), were collected from a commercial plantation, surface sterilized with 10 % bleach (0.5 % NaOCl), reduced in size, disinfected again with 2 % bleach, and rinsed three times with sterile distilled water under aseptic conditions. Shoot tip explants (0.5 cm in length) were excised and immersed in antioxidant solution. Half of the explants were cultivated on the medium recommended by Novak et al., and half on the medium described by Sandoval. Fifty shoot tips from in vitro plantlets were treated with 0.5 % (v/v) EMS for two hours at 30 °C, and rinsed three times with sterile distilled water. The explants were cultivated on MS + 1 mg/l BA and then transferred to MS + 4 mg/l BA. Material was transferred to test tubes with MS for root development. Rooted plantlets were hardened in a glasshouse for 12 weeks, placed in a nursery, and finally planted in the field at Los Diamantes Experimental Station. In this field location plants are exposed to natural inoculum and can be evaluated using the scale proposed by Stover as modified by Gaul. Considerable phenotypic variation was observed among the plants produced in vitro from EMS-treated shoot tips. After 11 months, 13 clones were selected for further evaluation due to their tolerance to black sigatoka. These clones were further propagated in order to establish a commercial planting for economic evaluation.

1. INTRODUCTION

According to the National Banana Corporation (CORBANA), last year Costa Rica exported 74.1 million boxes of bananas (18.14 kg each), produced on 28 296 hectares. One of the most striking problems in the banana and plantain production areas is control of black sigatoka, caused by Mycosphaerella fijiensis var. difformis. This disease was first reported in Costa Rica in October of 1979 [1, 2], and it has now spread throughout the banana and plantain production areas. Control of black sigatoka has been accomplished using fungicides. This practice, considerably increases the cost of production and environmental concerns.

The most effective way to solve problems with black sigatoka is through the use of varieties resistant to this disease. However, use of traditional plant breeding methods to produce resistant hybrids is very difficult in this generally seed sterile crop. Mutation induction is an alternative tool for improvement of banana cultivars and for development of black sigatoka resistant genotypes. The use of in vitro plant tissue/cell culture techniques make this approach possible. These are the reasons that the current project involving genetic improvement of banana and plantain through in vitro mutation breeding was started in Costa Rica.

2. MATERIALS AND METHODS

2.1. First step

Fifty corms of the banana clone "Gran Enano" (AAA), were collected from a commercial plantation. In the laboratory they were washed using tap water to eliminate soil residue. Corms were reduced in size by removal of leaves until they were about 8 cm. in length, disinfected in 10 % Clorox (0.525 % sodium hypochlorite) containing two drops of tween 20/100 ml for 20 minutes and then rinsed three times with sterile distilled water under aseptic conditions. Additional leaves were removed until the shoot was about 2 cm. long. They were then disinfected again in a 2 % Clorox solution containing two drops of tween 20/100ml, for 20 minutes. Explants were rinsed three times with sterile distilled water and further reduced in size to 0.5 cm in length. Shoot tips
were then immersed for 10 minutes in a sterile antioxidant solution containing L-cysteine (7.5 mg/l). Shoot tips were rinsed three times with sterile distilled water.

One-half of the total number of explants were cultured following the protocol recommended by Novak, Afza and Van Duren [3]. The explants were cultured individually in flasks (7 x 6 cm) containing 20 ml of MS basal medium (X1) supplemented with: 1 mg/l thiamine, 5 $\mu$M indole-3-acetic acid (IAA), 10 $\mu$M benzyladenine (BA), 40 g/l sucrose and 8 g/l agar. The other half of the explants were cultured on the medium described by Sandoval [4]. This medium consisted of MS basal medium (X2) supplemented with: 1 mg/l BA, 30 g/l sucrose and 7 g/l agar (Table I). Media pH were adjusted to 5.8 and media were sterilized by autoclaving at 121°C.

Table I. SUPPLEMENTS TO MEDIA

<table>
<thead>
<tr>
<th>Component</th>
<th>X-1</th>
<th>X-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>P</td>
</tr>
<tr>
<td>thiamine</td>
<td>1 mg</td>
<td>1 mg</td>
</tr>
<tr>
<td>IAA</td>
<td>5 $\mu$M</td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>10 $\mu$M</td>
<td>20 $\mu$M</td>
</tr>
<tr>
<td>IBA</td>
<td>-</td>
<td>1 $\mu$M</td>
</tr>
<tr>
<td>sucrose</td>
<td>40 g</td>
<td>40 g</td>
</tr>
<tr>
<td>agar</td>
<td>8 g</td>
<td>8 g</td>
</tr>
</tbody>
</table>

Basal medium Murashige and Skoog  
I : Initiation,  P : Proliferation,  E : Rooting

Flasks containing the explants were transferred to a growth room with a temperature of 28°C and 16 h (light) photoperiod. After 28 days, the explants on X1 initiation media were transferred to X1 proliferation medium (P) supplemented with 20 $\mu$M BA and kept on an orbital shaker at 50 rpm. The explants cultured on X2 initiation medium were transferred to X2 proliferation medium, supplemented with 5 mg/l of BA.

2.2. Second step

Fifty shoot tips produced in vitro were treated with a 5 % (v/v) solution of ethyl methanesulfonate (EMS) for two hours at 30°C as suggested by Novak et al. [3]. Shoot tips were then rinsed three times with sterile distilled water. The treated explants were cultured on MS medium supplemented with 1 mg/l BA for 30 days, and then transferred to MS medium supplemented with 4 mg/l BA as shown in Fig. 1. $M_1V_4$ plantlets (Fig. 1) were transferred to individual test tubes (150 x 25 mm) containing rooting medium (E) for root development.

Mutagenic Treatment

- $M_1V_1$ : 60 Days
- $M_1V_2$ : 30 Days
- $M_1V_3$ : 30 Days
- $M_1V_4$ : 30 Days
- Rooting : 30 Days
- Nursery : 30 Days

Fig. 1. Diagram of procedures used for production of mutated banana cv. Gran Enano.
2.3. Third step

Rooted plantlets were transferred to soil and maintained in the greenhouse for 12 weeks. A total of 1,234 plants were planted in the Diamantes Experimental Station in the Atlantic region; a location that has high concentrations of natural inoculum of black Sigatoka. No fungicides were applied to these plants and the field was placed under minimum agronomic management to observe the response of plants under natural epidemic condition.

Three evaluations were conducted on 1,104 plants at 9, 11 and 15 months after planting using the method of Stover as modified by Gaul [5]. During each evaluation the level of infection, and the specific leaf infected, were recorded. Plant vigor, fruit quality, and leaf shape were also examined. After evaluation some plants were selected and corms of them were taken for further micropropagation in the tissue culture laboratory.

3. RESULTS AND DISCUSSION

Fifty shoot tips from shoots produced in vitro, were treated with EMS and 1,600 plantlets were produced from them. After rooting, 1,234 plants were planted in the field. Of these, 1,104 reached an adult stage of growth (nine months old).

As observed by Novak et al. [6], many different mutant forms were observed after the M4V4 vegetative generation, mainly related to changes in leaf color, shape and distribution. Some dwarf mutants were also observed. After the first evaluation, 128 plants showed different levels of tolerance to black Sigatoka suggesting that the first infection occurred on the fifth or later leaf. From these 128 plants, nine mutants were identified as tolerant because they were infected only after the seventh or later leaf, and because infection at this stage permits the continued development of the plant and reasonable fruit production.

After the second evaluation four additional clones were selected as tolerant, for a total of 13 putative black Sigatoka-tolerant mutants. The third evaluation confirmed the previously identified tolerance in the selected mutants, 16 months after the plants were put into the field. These clones are being micropropagated now in order to establish a large-scale commercial planting for economic evaluation.

<table>
<thead>
<tr>
<th>Position of first infected leaf</th>
<th>Number of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st evaluation</td>
</tr>
<tr>
<td>7, 8 or 9</td>
<td>9</td>
</tr>
<tr>
<td>5 or 6</td>
<td>119</td>
</tr>
<tr>
<td>4</td>
<td>169</td>
</tr>
<tr>
<td>1, 2, 3</td>
<td>812</td>
</tr>
<tr>
<td>Total</td>
<td>1104</td>
</tr>
</tbody>
</table>

4. CONCLUSIONS

The method proposed by Novak et al. [3] to induce mutations in banana was used successfully in our experiments. Many clones showed changes in color, shape and orientation of their leaves. Some dwarf mutants were observed. Most importantly, different responses to black Sigatoka infection were observed among the mutagen-treated materials. Thirteen clones were selected as tolerant to this disease and they are now promising sources of germplasm for banana cultivation.
REFERENCES


RADIATION MUTATION INDUCTION
IN 'CHINA TIANBAO' BANANA

P. YANG, C. LI, F. WEI
Beijing Institute of Nuclear Engineering,
Beijing

W. HUANG, J. ZHENG, D. HUANG, R. HE
Zhangzhou Fruits and Vegetables Development Center,
Fujian

J. GUO, X. HUANG, B. CHEN, W. LIN
Zhangzhou Plant Quarantine and Monitoring Center,
Fujian
China

Abstract

This paper presents the results of irradiation experiments using 'China Tianbao' banana (Musa cv., AAA group) using in vitro culture methods. Explants of 3.0 to 6.0 mm long intact shoot tips, and shoot tips dissected longitudinally, were irradiated with $^{60}$Co. Differences in the radiation dose-survival response of the explants were observed among the different culture groups. The LD$_{50}$ dosages for intact shoot-tips and longitudinally dissected shoot-tips were 42.5 Gy and 38.69 Gy respectively. A regression equation of probability unit (Y) of death percentage of longitudinally dissected shoot-tips to dose logarithm (X) was established as $Y = 4.1703X - 1.6029$. In the irradiated materials which resumed growth after 45 days, statistical analyses suggested that there was no significant correlation between the rate of proliferation of treated materials and radiation dose, or between the proliferation rate and the technique used to prepare the materials (intact vs. longitudinally dissected). Experimental procedures were improved to decrease microbial contamination.

1. INTRODUCTION

'China Tianbao' banana is the dominant commercial banana cultivar of the Fujian Province located on the south-eastern coast of China. Its unique flavor is very much appreciated both at home and abroad. However, this cultivar is susceptible to hurricane damage and infection by banana bunchy top virus (BBTV) disease which greatly diminishes the yields of this banana cultivar. Therefore, improvement of 'China Tianbao' is important as it relates to the continued production of this cultivar in SE China.

Banana is a plant that is clonally propagated. It is difficult to breed commercial triploid bananas using classical hybridization methods due to the sterility of most clones. Genetic variation for many traits is limited in banana and selection of improved genotypes, obtained through controlled hybridizations, is very difficult. In many instances the only practical improvement method appears to be mutation breeding, and desirable mutation-induced characters can be produced, and subsequently maintained through vegetative propagation. Mutation induction can be used to improve one or more of the undesirable characteristics of this banana cultivar, while maintaining its superior characteristics. Mutation breeding using $^{60}$Co gamma rays can be an important means of banana improvement.

In the past, irradiation of suckers resulted in only limited mutation induction. Recently, with the development of biotechnological techniques, banana tissue culture methods can be used to produce the large populations of explants required for irradiation experiments [1, 2, 3]. In addition to its use in mutation induction experiments, tissue culture procedures can be used to generate so called somaclonal variants. The production of somaclonal variants may result in valuable genotypes.
The purpose of this research was to establish protocols for the improvement of 'China Tianbao' banana, by mutation breeding. The specific objectives of the project were the production of shorter plants with resistance or tolerance to banana bunchy top virus disease. Sensitivity of 'China Tianbao' banana to $^{60}$Co gamma rays, and methods of mutation breeding, are also discussed.

2. MATERIALS AND METHODS

The experimental materials were 'China Tianbao' banana (Musa cv., group AAA) which were obtained from a commercial orchard in Zhangzhou City, Fujian Province, P. R. of China. Healthy suckers were selected and used to initiate shoot-tip cultures. Shoot-tip cultures were propagated to provide sufficient shoot-tips for experimental use.

Five irradiation experiments were performed beginning in October 1990. In the first three experiments, in vitro shoot-tips (2.0 - 5.0 mm in length) were divided into two groups. Group A (intact shoot-tips) were placed in conical flasks. Group B (longitudinally dissected shoot-tips) were placed in sterile petri dishes with a few drops of sterile water. All culture vessels were sealed with parafilm. After irradiation with $^{60}$Co gamma rays, the materials were immediately transferred to culture medium. Unfortunately, these materials were contaminated, and were eventually discarded. The remainder of this report focuses on the two successful experiments.

The fourth experiment was begun on September 4th, 1992. The experimental materials were again divided into two groups; Group A (intact shoot-tips), and Group B (longitudinally dissected shoot-tips. Eleven levels of radiation were tested; 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 Gy, at a dose rate of 10 Gy/min. Sixty shoot-tips, 3.0 - 6.0 mm in length, were used in each irradiation treatment. In these experiments, the dissection and culture of the experimental materials were carried out aseptically in the laboratory. Five shoot-tip explants were placed in each culture bottle (conical flask). The culture medium was not changed in order to avoid possible contamination.

Based on the results of the fourth experiment, in which we determined the radiation dose response of the experimental materials to $^{60}$Co gamma rays, a fifth experiment was carried out the following October. The experimental materials were all dissected longitudinally and placed in conical flasks with solid medium. The irradiation doses were delivered at four levels: 0, 35, 45 and 55 Gy at a dose rate of 10 Gy/min. Two-hundred shoot-tip explants were used in each radiation dose treatment. Again, the culture medium was not changed after irradiation in order to minimize contamination.

During these previous two experiments, little contamination of experimental materials was observed and the irradiated shoot tips had a high survival rate. These results were satisfactory. Irradiated materials resumed growth 40 days after the irradiation treatment. Experimental materials were subcultured and rooted in vitro. After hardening, the cultured plantlets will be put into the field. Over 2 000 plants will be planted in a nursery to detect variants.

3. RESULT AND ANALYSIS

3.1. Radiation treatment and growth of explants

Table I illustrates the response (survival) of the treated experimental materials from the fourth experiment, which resumed growth after 45 days. In both the intact shoot-tip group (Group A) and the longitudinally dissected shoot-tip group (Group B) there was an apparent correlation between the radiation dose and the death of the shoot tips. There were also differences in the response of explants to the radiation dose when comparing the control group and the treated group. During the early stages, untreated explants became blackened. Treated explants did not display these symptoms immediately and they did not proliferate. Browning and blackening of the treated explants began fifteen days after irradiation. Examination of leaf primordia of surviving and dead
treated explants revealed them to be spongy. Some explants that eventually died developed one or two leaf primordia initially. This effect on leaf primordia might be explained by assuming that the radiation treatment had its greatest damaging effect on the cells located towards the middle of the apical meristem, with less damage occurring to the cells located away from the meristem. This phenomenon agrees with the results reported by Shi Yin Ping [4, 5]. Probably, the death of the explants is related to physiological defects caused by the radiation treatment.

Table I. GROWTH OF EXPLANTS AFTER EXPOSURE TO DIFFERENT DOSES OF IRRADIATION (45 DAY AFTER TREATMENT)

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (intact)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. dead</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>29</td>
<td>51</td>
<td>46</td>
<td>54</td>
<td>59</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>No. survived</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>52</td>
<td>31</td>
<td>9</td>
<td>14</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Proliferation</td>
<td>172</td>
<td>150</td>
<td>151</td>
<td>107</td>
<td>45</td>
<td>11</td>
<td>14</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Group B (longitudinally dissected)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. dead</td>
<td>2</td>
<td>3</td>
<td>10</td>
<td>11</td>
<td>24</td>
<td>40</td>
<td>54</td>
<td>56</td>
<td>57</td>
<td>58</td>
<td>60</td>
</tr>
<tr>
<td>No. survived</td>
<td>58</td>
<td>57</td>
<td>50</td>
<td>49</td>
<td>36</td>
<td>20</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Proliferation</td>
<td>130</td>
<td>135</td>
<td>100</td>
<td>109</td>
<td>76</td>
<td>26</td>
<td>10</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

3.2. Relationship between different radiation doses and treated materials

These dosage response data (Table I) were subjected to statistical analysis. The results of this analysis are shown in Table II.

Table II. STATISTICAL ANALYSIS OF RADIATION DOSE RESPONSE OF THE GROUP B (LONGITUDINALLY DISSECTED SHOOT-TIPS)

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>Percent dead plants*</th>
<th>Rate of death relative to control**</th>
<th>Logarithm dose (lgx)</th>
<th>Probability unit of percent death (y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.33</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>10</td>
<td>5.00</td>
<td>1.67</td>
<td>1.00000</td>
<td>2.8799</td>
</tr>
<tr>
<td>20</td>
<td>16.67</td>
<td>13.34</td>
<td>1.30103</td>
<td>3.8877</td>
</tr>
<tr>
<td>30</td>
<td>18.33</td>
<td>15.00</td>
<td>1.47712</td>
<td>3.9634</td>
</tr>
<tr>
<td>40</td>
<td>40.00</td>
<td>36.67</td>
<td>1.60206</td>
<td>4.6602</td>
</tr>
<tr>
<td>50</td>
<td>66.67</td>
<td>63.36</td>
<td>1.69897</td>
<td>5.3425</td>
</tr>
<tr>
<td>60</td>
<td>90.00</td>
<td>86.67</td>
<td>1.77815</td>
<td>6.1123</td>
</tr>
<tr>
<td>70</td>
<td>93.33</td>
<td>90.00</td>
<td>1.84510</td>
<td>6.2816</td>
</tr>
<tr>
<td>80</td>
<td>95.00</td>
<td>91.67</td>
<td>1.90309</td>
<td>6.3852</td>
</tr>
<tr>
<td>90</td>
<td>96.67</td>
<td>93.34</td>
<td>1.95424</td>
<td>6.4985</td>
</tr>
<tr>
<td>100</td>
<td>100.00</td>
<td>96.67</td>
<td>2.00000</td>
<td>6.8384</td>
</tr>
</tbody>
</table>

* : Number dead/60 x 100 %

** : (Percent dead) - (Value of control)
3.2.1. \( \text{LD}_{50} \) dose of the longitudinally dissected shoot-tips group

As shown in Table II, the correlation coefficient \((r)\) of logarithm dosage and percent explantage death was determined to be 0.9716. This is a high correlation.

The regression equation of probability unit \((Y)\) of percent death to log dose was \(Y = 4.1703X - 1.6029\). The significance of the F value was examined; \(F = 161.2083\) \((F_{0.01} = 11.26)\). F values were determined to be significant at the levels indicated in Table III. The regression equation \((Y = 4.1703X - 1.6029)\) was tenable (Figure 1).

Table III. ANALYSIS TABLE OF THE REGRESSION EQUATION OF DOSE LOGARITHM AND PROBABILITY UNIT OF DEATH PERCENTAGE

<table>
<thead>
<tr>
<th>Mutation source</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>(F)</th>
<th>(F_{0.01})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>1</td>
<td>15.8629</td>
<td>15.8629</td>
<td>161.2083**</td>
<td>11.26</td>
</tr>
<tr>
<td>Surplus</td>
<td>8</td>
<td>0.7875</td>
<td>0.0984</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total mutation</td>
<td>9</td>
<td>16.6504</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 1. The dose-response regression of percentage death of longitudinally dissected shoot-tips to radiation dose in logarithm.](image)

When the death rate reached 50 \%, the probability unit of percentage death was 5. If \(Y = 5\), and using the equation \(Y = 4.1703X - 1.6029\), then \(X = 1.5876\). The inverse log of \(X\) was the \(\text{LD}_{50}\) dose which equaled 38.69 Gy, e.g. the \(\text{LD}_{50}\) for the longitudinally dissected shoot-tip group was 38.69 Gy.

The curve of the relationship between percentage death and radiation dose is shown in Fig. 2. This dose response curve is close to a typical 'S' shaped curve. Thus, when the radiation doses are small, increases in the percentage of dead explants increase slowly. When the radiation doses are
in the mid-range of the curve, small increases in radiation result in more rapid increases in the percentage of dead explants. At the high end of the curve further increases in dosage illicit only slight increases in the percentage of explants that do not survive. This response of banana shoot-tips to irradiation with $^{60}\text{Co}$ gamma rays is similar to the reported responses of other plants to irradiation [6].

![Fig. 2. The dose-response curve of longitudinally dissected shoot-tips.](image)

3.2.2. LD$_{50}$ dose of intact shoot-tips

The results of experiments examining the radiation dose-response are shown in Table IV. From Table IV it can be seen that the correlation coefficient ($r$) of the log dose and the probability unit of percent death was 0.1793 ($r = 0.1793$ is $< r_{0.05} = 0.6319$). Thus a significant correlation does not exist and a regression equation was not calculated. From the curve in Fig. 3, the LD$_{50}$ dose of intact shoot-tips was 42.5 Gy. The shape of the curve was similar to that the curve in Fig. 2.

![Fig. 3. The dose-response curve of intact shoot-tips.](image)
### Table IV. STATISTICAL ANALYSIS OF RADIATION DOSE RESPONSE OF INTACT SHOOT TIPS

<table>
<thead>
<tr>
<th>Radiation dose(Gy)</th>
<th>Percent dead plants*</th>
<th>Relative rate of death**</th>
<th>Transformed log unit of death (%)***</th>
<th>Dose in log unit of death (lgy)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00000</td>
<td>2.6737</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
<td>1.00000</td>
<td>2.6737</td>
</tr>
<tr>
<td>20</td>
<td>13.33</td>
<td>13.33</td>
<td>14.33</td>
<td>1.47712</td>
<td>3.9331</td>
</tr>
<tr>
<td>30</td>
<td>48.33</td>
<td>48.33</td>
<td>49.33</td>
<td>1.60206</td>
<td>4.9825</td>
</tr>
<tr>
<td>50</td>
<td>85.00</td>
<td>85.00</td>
<td>86.00</td>
<td>1.69897</td>
<td>6.0803</td>
</tr>
<tr>
<td>60</td>
<td>75.67</td>
<td>75.67</td>
<td>76.67</td>
<td>1.77815</td>
<td>6.7290</td>
</tr>
<tr>
<td>70</td>
<td>90.00</td>
<td>90.00</td>
<td>91.00</td>
<td>1.84510</td>
<td>6.3408</td>
</tr>
<tr>
<td>80</td>
<td>98.33</td>
<td>98.33</td>
<td>99.33</td>
<td>1.90309</td>
<td>7.4573</td>
</tr>
<tr>
<td>90</td>
<td>98.33</td>
<td>98.33</td>
<td>99.33</td>
<td>1.95424</td>
<td>7.4573</td>
</tr>
<tr>
<td>100</td>
<td>98.33</td>
<td>98.33</td>
<td>99.33</td>
<td>2.00000</td>
<td>7.4573</td>
</tr>
</tbody>
</table>

*: No. dead/60 × 100 %

**: (Percentage dead plants) - (Value of control)

***: % dead was zero at 10 and 20 Gy. Thus, 1.00 was added to all the % dead values for ease of calculation.

3.2.3. Effect of radiation on the proliferation rate of the irradiated materials

F values were calculated and are shown in Table VI. F values were not significant. This indicates that the rate of proliferation was independent of the radiation dose, and that there was no significant difference between the rate of proliferation of longitudinally dissected and intact shoot-tips.

### Table V. RELATION OF PROLIFERATION MULTIPLE AND THE RADIATION DOSE

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group B**</td>
<td>2.8667</td>
<td>2.5000</td>
<td>2.5167</td>
<td>2.0577</td>
<td>1.4516</td>
<td>1.2222</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Group A***</td>
<td>2.2414</td>
<td>2.3604</td>
<td>2</td>
<td>2.2245</td>
<td>2.1111</td>
<td>1.3000</td>
<td>1.6667</td>
<td>1.5</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*: (Total number)/(Number surviving)

**: Intact shoot tips

***: Longitudinally dissected shoot tips

### Table VI ANALYSIS OF RELATIONSHIP AMONG TREATMENT PATTERN, RADIATION DOSE AND PROLIFERATION MULTIPLE

<table>
<thead>
<tr>
<th>Mutation source</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean of square</th>
<th>F</th>
<th>F_{0.05}</th>
<th>F_{0.05}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between doses</td>
<td>9</td>
<td>6.2430</td>
<td>0.6937</td>
<td>0.1034</td>
<td>5.35</td>
<td>3.18</td>
</tr>
<tr>
<td>Between treatments</td>
<td>1</td>
<td>0.0044</td>
<td>0.0044</td>
<td>0.0007</td>
<td>10.56</td>
<td>5.12</td>
</tr>
<tr>
<td>Error</td>
<td>9</td>
<td>60.3956</td>
<td>6.7106</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total mutation</td>
<td>19</td>
<td>66.6430</td>
<td>7.4087</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2.4. LD₅₀ dose of intact shoot-tips

The LD₅₀ dose for intact shoot tips was calculated to be 42.5 Gy. This was higher than the LD₅₀ dose (38.6 Gy) for longitudinally dissected shoot-tips. This suggests that the longitudinally dissected shoot-tips were more sensitive to ⁶⁰Co gamma rays, perhaps due to injury during dissection or damage to the meristem tissue.

4. DISCUSSION

4.1. Sensitivity and LD₅₀ dose of materials irradiated by ⁶⁰Co gamma rays

Experimental results indicate that there is an obvious difference in sensitivity to ⁶⁰Co of intact and longitudinally dissected explants. The longitudinally dissected shoot-tips were more sensitive than the intact shoot-tips and their LD₅₀ doses were 38.69 Gy and 42.5 Gy, respectively. The longitudinally dissected shoot-tips were considered to be more suitable for irradiation experiments.

Figure 3 which illustrates the results of the irradiation response of intact shoot-tips indicates that the values are dispersed. Through more rigorous statistical analysis [7, 8], the response appears to be neither linear nor a typical 'S' -shaped curve relationship. Thus the LD₅₀ dose of 42.5 Gy is only an estimate. In contrast, the LD₅₀ dose of longitudinally dissected shoot-tips was determined.

4.2. Effect of ⁶⁰Co gamma rays on proliferation rate of explants

The proliferation rate of explants was not correlated with either the radiation dose or the method of explant preparation (longitudinal dissection vs intact). It should be noted that the time required for proliferation was twice that required during the usual proliferation cycle. Based on the reports of Zhou Li Nong [9, 10, 11] and Li Bao Rong [12], the maximum proliferation rate of shoot-tips is generally reached after 15 days, and the proliferation multiple is highest at 20 days. The proliferation coefficient of shoot-tips that formed only one leaf primordia was much lower than that of young shoots. It remains to be determined whether or not the synchronization of proliferation was the result of a long growth time. It should also be mentioned that the size of explants (5.0 - 10.0 mm) normally used for propagation is larger than the size of explants (3.0 - 6.0 mm) used in these experiments.

4.3. Experimental method to avoid fungal contamination

Three experiments were carried out earlier during this research, two in Beijing and one in Fuzhou. During experiments, there were considerable problems with fungal contamination. The source of the fungal contamination may have been environmental, or the result of the long-distance transportation of experimental materials. It might be also related to the radiation source in the laboratory which was not well-suited for aseptic work. Therefore, in the present experiments, all the experimental operations were carried out in the in vitro culture laboratory. The practice of sealing with parafilm was replaced by using rubber plugs, and there was no reculture of explants to fresh medium immediately after the irradiation. These steps resulted in satisfactory control of fungal contamination.

4.4. Selection of mutants

During the period of growth following irradiation with ⁶⁰Co gamma rays, our observations were mainly focused on changes in leaf color and leaf shape. It was difficult to detect promising mutants at this stage. The selection of favorable mutant characters should emphasized in the nursery and in the field. Our research is presently focused on the examination of the radiation response of cultured banana tissues. Our work on the selections of gamma irradiated materials, fundamental investigations on the effects of irradiation on the resumption of growth, the time
period required for micropropagation, and observation of plants in the nursery and in the field, will continue.

REFERENCES


IMPROVEMENT OF BANANA (Musa cvs.) THROUGH IN VITRO CULTURE TECHNIQUES AND INDUCED MUTATIONS

S. H. SIDIQUI, A. KHATRI, I. A. KHAN, G. NIZAMANI, R. KHAN
Atomic Energy Agricultural Research Centre,
Tando Jam, Sindh, Pakistan

Abstract

Rapidly multiplying shoot tip cultures were established from meristem explants of banana (Musa cvs.). Banana cultivars were successfully propagated on modified Murashige and Skoog medium with 4.5 mg/l BAP. Roots were induced on plantlets cultured on 1/2 strength MS medium containing 0.20 mg/l IBA. Plantlets were acclimatized and transferred to soil for further evaluation. Proembryogenic calli were initiated from basal leaf sheaths and rhizome tissue cultured on SH medium with 6.63 mg/l dicamba. Protoplasts were isolated from rhizome tissue using an enzyme solution containing 3 % cellulase R-10, 1 % macerozyme R-10 and 1 % pectinase. Efforts are in progress to regenerate plants via somatic embryogenesis and from isolated protoplasts. A mutation induction experiment was carried out by irradiating vegetative shoot apices at 5, 10, 15 and 20 Gy using a ^60Co source. Radiosensitivity was assessed by determining the subsequent rate of shoot differentiation.

1. INTRODUCTION

Pakistan is a land of diverse ecology capable of producing a variety of horticultural crops. Banana is a major fruit crop in Pakistan. The cultivated area of banana in 1947 was about 117 hectares, with 365 tones of fruit production. Since that time the area has increased to about 23 100 hectares, with 205 700 tones of fruit production (Fig.1)[1]. The average yield of 8.88 MT/hectare is low when compared to other banana growing countries and these yields need to be increased by growing genetically improved cultivars with high yields and superior quality characteristics. The genetic improvement of banana by conventional breeding techniques is hampered due to pollen, ovule and seed sterility [2, 3].

Banana is a vegetatively propagated crop where new cultivars commonly originate through spontaneous mutations [4]. All significant cultivars are triploid and sterile. This has made the application of conventional breeding methods more difficult [5]. The recent and rapid spread of banana bunchy top virus (BBTV) in the province of Sindh has almost eliminated the cultivation of banana in this region as no cultivars resistant to BBTV are known to exist. Thus, breeding for resistance requires new strategies such as the use of in vitro culture techniques.

Tissue culture techniques have made a significant contribution to plant breeding and are especially useful in banana [6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16]. A combination of conventional breeding and biotechnology provides the necessary tools for the development of new clones resistant or tolerant to diseases [2] and for the elimination of virus [16]. Tissue culture facilitates mass propagation, conservation and easy movement of germplasm. Other techniques such as somatic embryogenesis and protoplast culture of economically important cultivars offer new opportunities for inducing genetic variability (somaclonal variation).

The induction of somatic mutations could contribute the broadening of genetic variation in banana [17]. These techniques were utilized successfully to develop the mutant clone GN60A [12]. Therefore investigations of biotechnological techniques, micropropagation, cell/tissue culture, protoplast culture, and induced somatic mutation, were initiated at AEARC, Tando Jam, to overcome our local problems with banana production.

In this paper we describe the use of the above techniques to develop a micropropagation system for rapid clonal propagation, for obtaining disease-free plants for nursery stock and for the production and isolation of banana somaclones and mutants with improved characteristics such as fruit quality and resistance to biological and environmental stresses.
2. MATERIALS AND METHODS

2.1. Micropropagation

Clones of banana (Musa cvs.) used in this study were Basrai (Dwarf Cavendish), Williams hybrid, SH3362, Magrabi, Hindi, Grand Nain, GN60A, Highgate and 30-29. Plant material was provided by Dr. F.J. Novak of Seibersdorf Laboratories, IAEA, Vienna, Austria, and Dr. M.K. Smith, Maroochy Horticultural Research Station, Queensland, Australia.

Suckers/corms consisting of shoot tips and rhizomatous base tissue were used as explant materials. Explants were initially washed in tap water and the outer layers of leaves were removed. Shoot apices with 2 - 4 leaf primordia were obtained and these were disinfected in 70 % ethanol (one minute), by immersion in 0.5 % sodium hypochlorite solution for 15 minutes, and then rinsed thoroughly in sterile distilled water. More leaves were removed until the remaining shoot tips were 1 cm at the base. Explants were cut into four equal pieces, and transferred aseptically into 150 x 25 mm test tubes containing Murashige and Skoog [18] medium supplemented with 1 mg/l thiamine-HCl, 0.88 mg/l indole-3-acetic acid (IAA), 2.25 mg/l benzylaminopurine (BAP), 40 gm/l sucrose and solidified with 8 gm/l bacto agar. Cultures were maintained in a growth chamber at 28 °C on a 16 h photoperiod. After 21 days of culture the shoots were cut longitudinally and transferred into solid, as well as liquid, MS medium containing 4.5 mg/l BAP for additional shoot multiplication. Cultures in liquid medium were placed on a horizontal gyratory shaker at 60 rpm. Shoot proliferation cultures were subcultured at 28 day intervals. For rooting, individual shoots were placed in half-strength MS medium containing 4 % sucrose and 0.20 mg/l indole-3-butyric acid (IBA). Rooted plantlets were transferred to jiffy pots, acclimated under controlled conditions, and subsequently transferred to the soil.

2.2. Callus induction

Rhizome and leaf sheath tissues were excised from in vitro shoot cultures, and used as explants for callus induction. The culture media used was comprised of macro- and micro-elements
of Schenk and Hildebrandt [19], Staba vitamins, 100 mg/l inositol, 40 mg/l cysteine-HCl, 40 g/l sucrose and 6.63 mg/l 3,6 dichloro-2 methoxy benzoic acid (dicamba) was used.

2.3. Protoplast culture

Leaf, leaf base and corm tissues were obtained from proliferating shoot cultures and were used for protoplast isolation. Tissues were digested with a protoplast isolation enzyme mixture (3 % cellulase R-10, 1 % Macerozyme R-10, and 1 % pectinase). Protoplast were separated from the enzyme mixture by filtration and centrifugation at 100 x g for 10 minutes. Protoplasts were washed in culture medium (SH salts + staba vitamins + 6.63 mg/l dicamba + 0.4 M mannitol), separated by centrifugation, and finally resuspended in wash medium. Protoplast viability was determined by fluorescein diacetate (FDA) staining and density was determined sing a counting chamber (hemacytometer). Cultured protoplasts were incubated at 25 °C in the dark.

2.4. In vitro mutagenesis

Shoot tips of cvs. Magrabi and GN60A were isolated from proliferating in vitro shoot cultures. Shoot tips were cut longitudinally, transferred to petri dishes containing a few drops of sterile distilled water, and irradiated with gamma rays from 60Co source at doses of 5, 10, 15 and 20 Gy. Irradiated shoot tips were then transferred to micropropagation medium. After 4 weeks, radiosensitivity was evaluated in terms of in vitro growth rate and subsequent shoot proliferation.

3. RESULTS AND DISCUSSIONS

3.1. Micropropagation

The color of explants cultured on medium containing IAA and BAP changed from creamy white to green within 7 - 10 days. Shoot tips initially produced one or few shoots within 10 to 15 days after culture initiation. After culture establishment, the explants were transferred to multiplication medium containing 4.5 mg/l BAP. Longitudinally splitting shoot tips stimulated shoot multiplication during subsequent subcultures on multiplication medium. Splitting of shoot tips stimulated multiple shoot formation. Reculture of split shoot tips to liquid medium greatly increased the rate of growth and the rate of shoot proliferation when compared with explants cultured on solid medium. The average number of shoots per cluster was 10 in liquid medium and six in solid medium. It was also observed that explants showed tendency to produce phenolic compounds which oxidized rapidly and resulted in the death of some explants. The use of cysteine-HCl (40 mg/l) inhibited oxidation.

Shoots (3 - 5 cm length) were separated and transferred to rooting medium. Roots initiation was observed 7 - 10 days after transfer. Plants with well developed root systems were transferred to jiffy pots containing perlite and irrigated daily with distilled water and twice a week with liquid MS media. After acclimatization for 1 - 2 weeks plants were exposed to natural greenhouse conditions for 8 - 12 weeks. Plants were then transplanted into the field.

Plants propagated by this procedure are growing vigorously in the field. Survival of plants during transfer from culture vessel to soil was 100 %. Plants are phenotypically identical to the parental clone and have not shown any symptoms of disease. The performance of these plants, in the field, is under evaluation.

3.2. Callus induction

Rhizome and leaf tissue explants proliferated proembryogenic callus within 3 - 4 weeks after culture on SH medium containing Staba vitamins and 6.63 mg/l dicamba. The callus produced on this medium frequently appeared to be morphogenetically competent i.e. was comprised of small, more or less isodiametric and cytoplasmically dense cells. Virtually all our initial attempts to induce and sustain vigorous callus proliferation from shoot tips or leaf base tissue were inhibited by severe explant browning. A combination of 6.63 mg/l dicamba and 1.1 mg/l thidiazuron (TDZ)
improved callus initiation and helped to reduce explant darkening. The use of cysteine-HCl also reduced oxidation.

Explants from diploid clone SH 3362 proliferated both callus and proembryonal structures. Explants from triploid clone GN60A formed semi-compact calli and occasionally proembryonal structures. These proembryogenic structures were transferred to regeneration medium. Shoot formations from these structures was not observed. Efforts are in progress to regenerate plants through the process of somatic embryogenesis.

3.3. Protoplast isolation and culture

Attempts to isolate banana protoplasts used an enzyme mixtures. Of the explants tested, corm tissue gave the highest yields of protoplasts. Protoplasts were not isolated successfully from leaf tissue. Average yields of protoplasts from corm tissue ranged from $2.5 \times 10^5$ to $8.7 \times 10^5$ protoplasts/gram fresh weight of corm tissue. Freshly isolated protoplasts were generally colorless and the population was heterogenous with respect to size. Undigested clumps of cells were not detected in purified protoplast preparations. Eighty percent of the protoplasts stained with FDA were highly fluorescent under UV light indicating a high level of viability immediately after isolation. These protoplasts were cultured on callus induction medium and remained viable up for 2 - 3 weeks after isolation. Cell wall formation was observed 2 days after culture initiation. However, no callus colony formation was observed. Efforts are in progress to induce callus initiation and regenerate plantlets from isolated protoplasts.

3.4. In vitro mutagenesis

No adverse effects on explant viability, growth or shoot formation were observed after irradiation with 5 to 20 Gy (Table I). Shoot formation appeared to be slightly stimulated by low doses of irradiation. Shoot proliferation occurred from small nodular growths. The nodules contained small buds which either remained dormant or eventually developed into green shoots. Regenerated plantlets were readily established under non-aseptic growth conditions and a number of plants have been established under field conditions. Observations on vegetative growth, suckering, flowering and fruiting will be recorded to maturity for the mutants having desirable agronomic characteristics.

Table I. SHOOT REGENERATION IN SUBCULTURED TISSUES DERIVED FROM UNIRRADIATED AND IRRADIATED EXPLANTS. (DATA EXPRESSED AS AVERAGE NUMBER OF BUDS AND/OR SHOOTS PER CLUSTER)

<table>
<thead>
<tr>
<th>No. of subcultures</th>
<th>Irradiation dose (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Clone Magrabi</strong></td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td>5.12</td>
</tr>
<tr>
<td>2nd</td>
<td>6.75</td>
</tr>
<tr>
<td><strong>Clone CN60A</strong></td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td>7.75</td>
</tr>
<tr>
<td>2nd</td>
<td>8.37</td>
</tr>
</tbody>
</table>

Acknowledgement

The authors are indebted to Dr. K. A. Siddiqui, Director, AEARC, Tandojam, and Dr. A. R. Azmi (Ex-Director) for the support to establish a tissue culture laboratory. Also IAEA and Dr. F.
REFERENCES


BIBLIOGRAPHY


WONG, W.C. In vitro propagation of bananas (Musa spp.): Initiation, Proliferation and development of shoot tip culture on defined media. Plant Cell, Tissue and Organ Culture (1986) 6: 159-166.
EFFECT OF TOXIC FILTRATES OF Fusarium oxysporum f. sp. cubense ON THE DEVELOPMENT OF BANANA (Musa cvs.) SHOOT TIPS

B.M.J. MENDES, B.I.P. RODRIGUES, A. TULMANN NETO
Centro de Energia Nuclear na Agricultura,
Universidade de Sao Paulo,
Piracicaba, Brazil

Abstract

The objective of this study was to evaluate the effect of toxic filtrate of Fusarium oxysporum f.sp. cubense culture on the in vitro development of banana (Musa) shoot tips. Shoot tips from the varieties Nanicao (AAA - resistant to Panama disease) and Pisang mas (AA - susceptible to Panama disease) were isolated and cultivated in culture medium containing different concentrations of toxic filtrate of Fusarium culture. In the first assay, the concentrations of toxic filtrate tested were 0, 3, 6, 9 and 12.0 % (v/v) and then, based on these results, a second assay was conducted using 0, 1.5, 3, 4.5 and 6 % of Fusarium culture filtrate. Five replicates per treatment were used. Each replicate consisted of an erlenmeyer flask containing 15 ml of culture medium and five explants. The flasks were incubated on a shaker at 50 rpm and a daylength of 16 hours. After four weeks total fresh weight, fresh weight of shoots, and the length of shoots, were measured. No difference in the response of resistant and susceptible varieties was detected under low (0-3 %) or high (> 6 %) concentrations of Fusarium filtrate. At intermediate concentrations (3 - 4.5 %) it was possible to discriminate between the resistant and susceptible varieties based on the measured parameters, primarily total fresh weight and fresh weight of shoots. These data suggest that the use of tissue culture and Fusarium culture filtrates may be used together to study host-pathogen interactions.

1. INTRODUCTION

Fusarium Wilt or Panama Disease, caused by Fusarium oxysporum f.sp. cubense in banana, is one of the most destructive tropical diseases [1]. The microorganism was initially identified as a pathogen on clones of Gros Michel (AAA) and on dessert bananas such as the Brazilian varieties Maga (AAB) and Prata (AAB). Recently, the disease has also been reported to attack varieties of the Cavendish group (AAA) and to occur in the subtropical regions of Australia, South Africa and Taiwan [2].

Tissue culture has been used as tool to study diseases of banana. Sun and Su [3] used banana plantlets grown in vitro to determine races and pathogenicity of Fusarium isolates, and for selection for resistance to the disease; Mourichon et al. [4] inoculated banana plantlets with Mycosphaerella fijiensis and demonstrated a culture medium containing different concentrations of Fusarium filtrate. At intermediate concentrations (3 - 4.5 %) it was possible to discriminate between the resistant and susceptible varieties based on the measured parameters, primarily total fresh weight and fresh weight of shoots. These data suggest that the use of tissue culture and Fusarium culture filtrates may be used together to study host-pathogen interactions.

Toxic filtrate of Fusarium culture has been used in the selection for disease resistance in several crops including potato [6], alfalfa [7] and carnation [8]. In banana, Epp [9] reported an inability to establish a sensitivity test with plantlets or leaf discs using toxic filtrate from cultures of Fusarium oxysporum f.sp. cubense or fusaric acid, the main component of the toxic filtrate. In assays with leaf discs, differences were found when the discs were placed in contact with either fusaric acid or toxic filtrate, an indication that the filtrate contains toxic components in addition to fusaric acid [10]. Because of the importance of understanding the pathogen elicitors and the response of the host to the pathogen at a cellular level, it was considered essential to more precisely evaluate the effect of toxic filtrate of F. oxysporum f.sp. cubense on the in vitro development of banana shoot tips.
2. MATERIALS AND METHODS

2.1. Plant materials

The assays were carried out with banana plantlets of the varieties Nanicao (AAA - resistant to F. oxysporum f.sp. cubense) and Pisang mas (AA - susceptible to F. oxysporum f.sp. cubense), which were obtained from in vitro culture. Cultures of these varieties were initiated from shoot tips, and were subsequently propagated from lateral shoots on culture medium containing MS (Murashige & Skoog) salts [11], to which 30 g/l of sucrose, 10 mg/l of thiamine, 5 mg/l of benzylaminopurine (BAP) and 8 g/l of agar were added.

2.2. Fungal culture filtrate

In order to obtain toxic filtrate of F. oxysporum f.sp. cubense, the fungus was cultivated in erlenmeyer flask containing Czapek-Dox (CD) liquid culture medium, for a period of 21 days. After that time period the culture was filtered through a Millipore filter (0.22 μm) and the filtrate was stored in a freezer.

2.3. Toxic media

The basic growth media consisted of MS inorganic salts [11] supplemented with B5 vitamins [12], cysteine (40 mg/l), sucrose (40 g/l), N6 2-isopentenyladenine (2-iP) (1 mg/l) and indole-3-butyric acid (IBA) (0.02 mg/l). In the first assay, filter sterilized toxic filtrate was added to the medium, after autoclaving, to provide final concentrations of 0, 3, 6, 9 and 12 %. In the second assay, toxic filtrate was tested at concentrations of 0, 1.5, 3.0, 4.5 and 6.0 %. The media (15 ml) were poured into 250 ml erlenmeyer flasks. Concentrations of toxic filtrate were calculated based on the concentration of fusaric acid in the filtrate, which was determined using a spectrophotometer.

2.4. Culture procedure

Shoot tips were isolated from banana plantlets growing in vitro. Each assay consisted of five replicates per treatment. Five explants were cultured per flask. The flasks were placed in a growth room maintained at 27 °C and illuminated with cool-white fluorescent light bulbs to provide a daylength of 16 hours. Cultures were evaluated after four weeks of incubation, by measuring total fresh weight of the plantlets, the fresh weight of the shoots, and the length of the shoots.

3. RESULTS AND DISCUSSION

The use of tissue culture techniques to study host-pathogen interactions, and in the selection for resistance to diseases, has been reported for several host-pathogen systems including: tobacco-Phytophthora parasitica var. nicotianae [13, 14], potato-Phytophthora infestans [6, 15], alfalfa-Phytophthora megasperma [16], alfalfa-Fusarium oxysporum f.sp. medicaginis [7, 17], potato-Pseudomonas solanacearum [18] and elm-Ceratocystis ulmi [19]. If the pathogen produces substances that are toxic to the host, the selection of plant material resistant to the pathogen may generally be achieved by addition of the toxin to the culture medium used to grow cells from the host in vitro.

In the case of banana the study of diseases and the selection for resistance have been associated with tissue culture techniques in at least two diseases: Panama disease [9] and Black Sigatoka [5], which are caused by pathogens that produce substances toxic to the host.

It is evident, from the results of the first assay in our study, that the addition of toxic filtrate of F. oxysporum f.sp. cubense to the culture medium interferes with the growth of the banana shoot tips (Table I). It was not possible, however, to observe differences between the resistant (Nanicao) and susceptible (Pisang mas) varieties under the concentration used in this assay. When the concentration of toxic filtrate was 6 % or more, shoot tips of both varieties did not develop, indicating that the concentrations tested (0 - 12 %) were not adequate for the in vitro differentiation.
of varieties based on their known reaction to Panama disease. The definition of the percentage of toxic filtrate utilized depends on the concentration of the toxic substances found in the filtrate, and this varies according to the pathogen and the conditions of the culture. In the case of *F. oxysporum* f.sp. *cubense,* fusaric acid is apparently the main component of the culture filtrates. It is known that concentrations of fusaric acid higher than 10^-5 M inhibit the growth of banana shoot tips [9] and that 0.8 mM of fusaric acid in the culture medium was used for selection of barley callus resistant to *Fusarium* [20]. In the host-pathogen systems described in the scientific literature, the percentages of toxic filtrate added to the culture medium range from 0 - 50 % for carnation-*F. oxysporum* f.sp. *dianthi* [8] and elm-*Ceratocystis ulmi* [19] and from 0 - 25 % for alfalfa-*F. oxysporum* f.sp. *medicaginis* [7]. In those studies, the percentage of filtrate chosen for selection assays were 20, 50 and 7.5 %, respectively. The low amount of toxic filtrate of *Fusarium* required in culture medium, relative to the other pathogens, is probably due to the high concentration of fusaric acid in these filtrates. In the potato-*Alternaria solani* system [21] 0.8 - 3.2 % of culture filtrate were used to stimulate the regeneration of shoots from callus.

Table I. EFFECT OF TOXIC FILTRATE OF *F. oxysporum* f. sp. *cubense* ON THE DEVELOPMENT OF BANANA SHOOT TIPS. FIRST ASSAY. AVERAGE OF 5 REPLICATIONS.

<table>
<thead>
<tr>
<th>Filtrate conc. (%)</th>
<th>Plant fresh weight (g)</th>
<th>Shoot fresh weight (g)</th>
<th>Shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nanicao</td>
<td>P.mas</td>
<td>Nanicao</td>
</tr>
<tr>
<td>0</td>
<td>0.83A</td>
<td>0.53A</td>
<td>0.74A</td>
</tr>
<tr>
<td>3</td>
<td>1.22A</td>
<td>0.39A</td>
<td>0.96A</td>
</tr>
<tr>
<td>6</td>
<td>0.26BC</td>
<td>0.05B</td>
<td>0.25B</td>
</tr>
<tr>
<td>9</td>
<td>0.17C</td>
<td>0.04B</td>
<td>0.17B</td>
</tr>
<tr>
<td>12</td>
<td>0.13C</td>
<td>0.03B</td>
<td>0.13B</td>
</tr>
</tbody>
</table>

Values followed by different letters in the same column are significantly different by Tukey's test (5 %).

Table II. EFFECT OF TOXIC FILTRATE OF *F. oxysporum* f. sp. *cubense* ON THE DEVELOPMENT OF BANANA SHOOT TIPS-SECOND ASSAY. AVERAGE OF 5 REPLICATIONS.

<table>
<thead>
<tr>
<th>Filtrate conc. (%)</th>
<th>Plant fresh weight (g)</th>
<th>Shoot fresh weight (g)</th>
<th>Shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nanicao</td>
<td>P.Mas</td>
<td>Nanicao</td>
</tr>
<tr>
<td>0</td>
<td>1.33A</td>
<td>0.69A</td>
<td>1.02A</td>
</tr>
<tr>
<td>1.5</td>
<td>1.09A</td>
<td>0.58AB</td>
<td>0.79AB</td>
</tr>
<tr>
<td>3.0</td>
<td>1.21A</td>
<td>0.44ABC</td>
<td>0.84ABC</td>
</tr>
<tr>
<td>4.5</td>
<td>0.82A</td>
<td>0.25C</td>
<td>0.51B</td>
</tr>
<tr>
<td>6.0</td>
<td>0.31B</td>
<td>0.20C</td>
<td>0.30C</td>
</tr>
</tbody>
</table>

Values followed by different letters in the same column are significantly different by Tukey's test (5 %).
Fig. 1. Effect of toxic filtrate on the reduction of the total fresh weight (A), shoot fresh weight (B) and shoot length (C) of banana shoot tips.
In the second assay, intermediate concentrations of toxic filtrate in the culture media (0 - 6 %) were tested. In this assay a difference between the varieties Nanicao and P. mas was observed at toxic filtrate concentration of 3 - 4.5 % (Table II). Shoot length was not statistically different. It can be observed from Fig.1 that both varieties responded similarly at low (0 - 1.5 %) and high (6.0 %) concentrations of toxic filtrate. However, at intermediate concentrations (3.0 - 4.5 %) there was a marked effect of the toxic filtrate on the development of shoot tips of the susceptible variety. The total fresh weight of Pisang mas plants cultured in medium containing 3 % toxic filtrate was 63 % of that of the control whereas for the resistant variety Nanicao the total fresh weight was 90 % of the control. When the concentration of toxic filtrate in the culture medium was 4.5 %, total fresh weights of the varieties Nanicao (resistant) and Pisang mas (susceptible) were 61 % and 36 % of the control cultures, respectively. The other traits followed a similar pattern.

The possibility of discriminating between banana varieties in *in vitro* tests using toxic filtrate of *F. oxysporum* f.sp. *cubense* allows the utilization of tissue culture techniques to determine both the pathogenicity of the isolates, the host biochemical responses to toxic substances produced by the pathogen, and in selection for disease resistance. These factors enable the rapid and accurate assessment of resistance using a large number of plants in a small laboratory space.

REFERENCES


IN VITRO MUTATION BREEDING FOR THE DEVELOPMENT OF BANANAS WITH RESISTANCE TO RACE 4, FUSARIUM WILT (Fusarium oxysporum f. sp. cubense)

M. K. SMITH, S. D. HAMILL, P. W. LANGDON
Queensland Department of Primary Industries,
Maroochy Horticultural Research Station,
SCMC, Nambour, Queensland

K. G. PEGG
Queensland Department of Primary Industries,
Division of Plant Protection,
Meiers Road, Indooroopilly, Queensland

Australia

Abstract

In 1985 the Queensland Department of Primary Industries initiated an in vitro mutation breeding program for banana improvement. The primary objective was to develop a banana that was resistant to race 4, Fusarium wilt which could effectively substitute for the Cavendish cultivars currently grown. Micropropagated bananas were exposed to gamma radiation from a $^{60}$Co source and the LD$_{50}$ was established at 40 Gy. However, the optimal dose selected for irradiating explants was 20 Gy. At this dosage visual changes were apparent and plant survival, at 73%, was sufficiently high to make the technique practical on a larger scale. Over 20,000 plants have been evaluated in the field at our race 4 screening site and nursery at Wamuran in southeastern Queensland. Of the irradiated plants examined only the putative mutants from Dwarf Parfitt, an extra-dwarf variety of Cavendish, show potential as a race 4 resistant Cavendish cultivar. Plants are intermediate in height between Dwarf Cavendish and Williams, the industry standard. Preliminary results indicate the putative mutants are faster cycling and more cold tolerant than Williams. More importantly they also appear to retain the resistance to race 4 shown by the mother plant, Dwarf Parfitt.

1. INTRODUCTION

Fusarium wilt of bananas, caused by *Fusarium oxysporum* f.sp. *cubense*, is recognized as one of the most destructive plant diseases in the world [1, 2]. The collapse of the banana export trades in the 1940's and 1950's, based on the susceptible cultivar 'Gros Michel', was saved only by replanting with resistant Cavendish cultivars. In 1981 another race of Fusarium Wilt, race 4, was identified which was capable of attacking Cavendish bananas [1]. The inability to control this disease by chemical or cultural practices and the lack of resistance in commercially acceptable cultivars means that development of bananas with resistance to race 4, Fusarium wilt is of high priority in banana improvement.

Cavendish are sterile, parthenocarpic triploids and, as such, have not featured in conventional breeding programs. The synthesis of new hybrids which can substitute for Cavendish also has its problems [3]. The difficulty associated with banana breeding can best be summed up by the fact that despite over 70 years of continuous breeding efforts, no new banana cultivar that is commercially acceptable as a Cavendish replacement has been produced. In fact, bananas are one of very few crops in which only clones derived from natural somatic mutations are cultivated.

Due to the difficulty and costs associated with conventional banana breeding, alternative strategies for genetic improvement have been actively pursued. One such strategy involves mutation induction. Mutation induction has become an established tool in plant breeding with hundreds of improved varieties having been released to growers from many different crop species [4]. The main advantage of mutation induction in a vegetatively propagated crop, such as banana,
is the ability to change one or a few characters of an otherwise outstanding cultivar without altering the remaining and often unique part of the genotype. This is not yet possible using conventional approaches to banana improvement. A summary of the results of an in vitro mutation breeding program for the development of a race 4 resistant Cavendish replacement, suitable for the subtropical conditions of south-east Queensland and northern New South Wales, is presented.

2. MATERIALS AND METHODS

Strains of *Fusarium oxysporum* f.sp. *cubense* were characterized by determination of vegetative compatibility groups [5], by analysis of volatile components produced on a steamed rice substrate [6] and by RAPD-PCR analysis.

Conditions for the initiation and growth of micropropagated bananas have been described by Hamill *et al.* [7] and Smith and Drew [8]. Briefly, Williams (AAA), New Guinea Cavendish (AAA) and Dwarf Parfitt (AAA) were initiated on a Murashige and Skoog [9] basal medium supplemented with 10 μM benzylaminopurine, 2 % sucrose and 0.8 % Difco-Bacto agar. This medium also supported rapid shoot multiplication. Cultures were incubated at 28 °C with a 16 h photoperiod. Cool white fluorescent tubes provided a light intensity at the culture surface of 80 μE m⁻² s⁻¹. Before acclimatisation in the glasshouse, plants were subcultured to a hormone-free medium for root initiation and for the development of vigorous, single shoots. Plants were established in the field when they reached a height of 20 cm in 2.5 l plastic bags.

The irradiation procedure involved the transfer of explants, containing the shoot tip and ensheathing leaf bases obtained from in vitro grown plantlets, to Petri dishes which contained a multiplication medium. Cultures were incubated for 7 days before exposure to gamma radiation from a 60Co source. The doses used were 0, 10, 20, 30, 40 at 50 Gy. Dosage was calculated using a Fricke dosimeter [10]. Plants were then subcultured for two or three cycles before disease resistance screening. Smith *et al.* [11] described the Fusarium wilt race 4 screening experiments which involved: (1) the use of *Fusarium* culture filtrates in vitro, (2) root dip inoculations and (3) direct planting in a *Fusarium* infested field site at Wamuran (latitude 27 °S) in south-eastern Queensland. Any survivors from the first two screening trials were also tested in the field. The site at Wamuran was a commercial block of Cavendish that first became infested with race 4 during 1976. The pathogen eventually spread and by 1984 the block had become commercially unviable. The site was leased by QDPI for development as a race 4 screening site and nursery. Levels of inoculum were maintained and increased by redistributing infected material on site. The *Fusarium* wilt population was intensively sampled and characterized by VCG and volatile analysis.

Plants that survived for one or two harvests without developing any symptoms of Fusarium wilt were divided into suckers and bits and replanted in infested sites. This was usually repeated for a second and third cycle to overcome the possibility of 'escapes' and to verify that unique clones may have arisen following micropropagation and irradiation.

3. RESULTS AND DISCUSSION

3.1. Characterization of strains of Fusarium wilt

The designation of race 4 is given to any Cavendish resistance-breaking strain of *Fusarium oxysporum* f.sp. *cubense* (FOC). However, these strains (biotypes) may be of separate origin and they may differ in virulence and even host range. In an international breeding or selection programme the precise classification of the pathogen used for screening for resistance must be known in relation to isolates of the pathogen from other areas.

Previously pathogenicity tests using a limited set of differential cultivars were the major means for differentiating the races of Fusarium wilt. Two pathotypes, races 1 and 2, were distinguished by their pathogenicity on Gros Michel and Bluggoe, respectively. Stover [12] further separated race 1 isolates into yellowing and non-yellowing clones based on the symptoms produced on Gros Michel. Other techniques have now been used to reliably identify genetic diversity within the
fungus. One approach has been to use vegetative compatibility which is based on the genetics of the fungus rather than the host-pathogen interaction. Strains of fungi which are able to form heterokaryons are vegetatively compatible and comprise vegetative compatibility groups (VCGs). Eleven VCGs have been characterized in FOC from a world-wide collection [13]. Using a number of isolates from south-eastern Queensland and northern New South Wales, vegetative compatibility studies have confirmed that Australian race 1 and 4 isolates belong to separate VCGs [5]. In Australia, race 4 is composed of three VCGs (VCG 0120; VCG 0129; VCG 01211), while race 1 isolates belong to two VCGs (VCG 0124; VCG 0125).

Another criteria which has been used to identify variation among Fusarium wilt isolates was the production of volatiles on steamed rice. Stover [14] cultured isolates on steam-sterilized rice and classified ‘odoratum’ or ‘inodoratum’ isolates based on their production of a strong volatile odour. Moore et al. [6] found that production of volatiles on steamed rice by Australian isolates correlated well with race and VCG. All race 4 isolates produced distinct volatile odours which gave characteristic gas chromatograms where the number of peaks equated to VCG. Similar chromatograms were obtained for race 4 isolates from Taiwan and South Africa. All race 1 isolates and race 2 isolates (VCG 0128) tested did not produce odorous volatile compounds and gave chromatograms without significant peaks. Chromatographic analysis differentiated qualitatively between volatile odours produced by isolates in the race 4 VCGs 0120 and 0129. Recently RAPD-PCR has been used to analyze genetic variation within FOC and results have been in close agreement with previous classifications based on host range, vegetative compatibility and chromatogram profiles of volatile compounds (S. Sorensen, pers. comm.).

3.2. Effect of gamma irradiation on in vitro growth of Cavendish cultivars

The LD$_{50}$ for micropropagated Williams was approximately 40 Gy, however shoot multiplication and general vigour of plantlets was poor (Fig. 1). The optimal dose range was 20 Gy and was used for the other Cavendish cultivars in this program. At this dosage visual changes were apparent and plant survival, at 73 %, was sufficiently high to make the technique practical on a larger scale. The radiosensitivity of the Cavendish cultivars used in these experiments compares well with that found by Novak et al. [15] for Grand Nain.

3.3. Determination of race 4, Fusarium wilt resistance in the Musa genepool

In addition to mutation breeding trials we have also evaluated a range of Musa species, cultivars and accessions for resistance to race 4 at our screening site at Wamuran. These plants were obtained from local and overseas germplasm collections and breeding programs. Included in this material was Musa germplasm collected from Papua New Guinea [16]. Currently 368 accessions have been screened and of these, only 6 accessions have shown resistance to Fusarium wilt race 4 (Table 1). They included two wild species, Musa ornata and M. velutina; two superior breeding diploids from the FHIA breeding program, SH-3362 and SH-3142; and Dwarf Parfitt, an extra-dwarf Cavendish variety. Mysore was susceptible when young but tolerated race 4 as the stool matured.

Though all of these plants showed good levels of resistance, they were not immune to Fusarium wilt. Under extreme conditions of cold and drought, as existed in southeastern Queensland in 1991, even some plants from this group became infected and showed symptoms of the disease. In fact, immunity is not a realistic goal to aim for in a Fusarium wilt breeding program in banana because regardless of the pathotype or cultivar used, the fungus is able to penetrate and established in the vascular system of the root [1]. Resistance can breakdown under periods when the plants are stressed and the pathogen is active. However if the level of resistance observed from these accessions can be expressed in a suitable commercial cultivar, than the possibility of serious epidemics of the disease may be considerably reduced. We believe Fusarium wilt can be effectively managed with resistant cultivars.
Vigour of surviving plantlets was rated on a scale of 1 to 5: (1) little to no growth, chlorosis and necrosis of leaves to (5) excellent growth and vigorous, green shoots. Clone 5 [ ], Clone 11 [ ], Clone 99 [ ].

**Fig. 1.** Effect of gamma irradiation on in vitro survival and vigour of selected Williams clones.
Table I. **Musa SPECIES AND CULTIVARS CONSIDERED TO SHOW RESISTANCE TO RACE 4 FUSARIUM WILT** (*Fusarium oxysporum* f.sp. *cubense*).

<table>
<thead>
<tr>
<th>Species/Cultivar</th>
<th>Genotype</th>
<th>Origin</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dwarf Parfitt</td>
<td>AAA</td>
<td>Local selection from New South Wales</td>
<td>Member of Cavendish subgroup. 1 m at bunch emergence. No commercial value</td>
</tr>
<tr>
<td>SH-3362, SH-3142</td>
<td>AA</td>
<td>Improved diploids from FHIA breeding program</td>
<td>Important as male, diploid parents but of no immediate commercial use</td>
</tr>
<tr>
<td>Mysore</td>
<td>AAB</td>
<td>Imported into Queensland from Jamaica and Honduras, originally from Indian subcontinent</td>
<td>Dessert fruit, moderate yielding</td>
</tr>
<tr>
<td>Musa ornata, M. velutina</td>
<td></td>
<td>Local selections from New South Wales</td>
<td>Ornamental bananas, colourful bracts</td>
</tr>
</tbody>
</table>

Based on observations from 368 accessions grown on heavily-infested soils at Wamuran in south-eastern Queensland.

### 3.4. Reaction of gamma irradiated Cavendish cultivars to race 4, Fusarium wilt

Two strategies were adopted for the *in vitro* mutation breeding program. The first approach involved irradiating the race 4 susceptible Cavendish cultivars, Williams and New Guinea Cavendish, to improve resistance but retain their agronomic characteristics. The second approach involved irradiating the agronomically inferior Cavendish cultivar, Dwarf Parfitt, to improve performance but retain resistance. Over 20,000 plants have been evaluated in the field and only the putative mutants derived from Dwarf Parfitt show the most promise as a race 4 resistant Cavendish replacement under the subtropical conditions existing in Queensland and New South Wales.

The first strategy of irradiating superior, but disease susceptible, Cavendish cultivars has yielded a few promising lines but a markedly improved level of resistance has not been observed. Planting material collected from the surviving plants, including the few that survived culture filtrate and root dip inoculation experiments, has been replanted for a second, third, and now for a fourth cycle of evaluation. Of the few remaining plants, all are characterized by a dwarf, robust stature (average height of 1.9 m at bunching). Unfortunately these plants are also prone to ‘choking’, a phenomenon where the bunch fails to emerge fully from the crown of the plant.

The second strategy was that of irradiating Dwarf Parfitt, an extra-dwarf Cavendish banana that has shown a high level of resistance to race 4. Unfortunately Dwarf Parfitt are very small, with an average height of 1.0 m at bunching, and are extremely prone to ‘choking’ (Fig. 2). Following irradiation of approximately 500 explants at a dose of 20 Gy, 35 M1V3 plants were recovered which possessed improved characteristics. Plants were larger, they bunched earlier, yield was considerably increased and choking was eliminated (Fig. 2, Table II). They were also apparently more cold tolerant than standard Cavendish cultivars. More importantly they also appeared to retain the resistance to race 4 shown by the mother plant, Dwarf Parfitt.
Fig. 2. Irradiated banana plants established in the field. (a) Dwarf Parfitt; (b) putative mutant of Dwarf Parfitt following gamma irradiation.
Table II. PRELIMINARY GROWTH AND YIELD DATA OF DWARF PARFITT AND ITS PUTATIVE MUTANT IN SOUTHEASTERN QUEENSLAND.

<table>
<thead>
<tr>
<th></th>
<th>Dwarf Parfitt</th>
<th>'Parfitt'</th>
<th>Williams</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height at bunching (cm)</td>
<td>104</td>
<td>182</td>
<td>203</td>
</tr>
<tr>
<td>Bunch weight (kg)</td>
<td>8.2</td>
<td>23.2</td>
<td>22.1</td>
</tr>
<tr>
<td>No. of hands/bunch</td>
<td>9.0</td>
<td>10.3</td>
<td>10.4</td>
</tr>
<tr>
<td>Planting to harvest (months)</td>
<td>19.8</td>
<td>16.3</td>
<td>17.9</td>
</tr>
</tbody>
</table>

Values are the averages from approximately 20 plants. The data for Williams was taken from non-infested sites as Williams succumbed to Race 4 Fusarium wilt in the original site.

We are still at an early stage in the evaluation of this material and we wish to determine the genetic stability of this material and make further comments on the nature of the resistance. It is interesting that Fusarium wilt of Cavendish is most prevalent in the subtropical regions of the world [2] where periods of cold and drought may place plants under stress. Leaves of our standard Cavendish varieties are typically more yellow that Dwarf Parfitt following winter (photoinhibition-induced chlorosis) and carry fewer photosynthetically competent leaves. We have speculated that the 'weakened' Cavendish cultivars are therefore most susceptible following winter and unable to prevent invasion of the fungus into the corm during the spring when the disease can reach almost epidemic proportions. By developing a greater understanding of the physiological mechanisms of resistance there is an opportunity that a reliable screening technique can be developed and applied either in culture or in the nursery to screen the large numbers of plants produced from mutation or conventional breeding programs. The lack of a suitable small plant screening technique continues to be one of the major obstacles in effectively breeding for Fusarium wilt resistance.

Acknowledgements

We thank L. Pedwell for his support and the use of his land as a race 4 screening nursery. The funding support of the Queensland Banana Industry Protection Board, the Horticultural Research and Development Corporation and the Australian Centre for International Agricultural Research is gratefully acknowledged.

REFERENCES


SOMATIC EMBRYOGENESIS IN BANANAS AND PLANTAINS
(Musa CLONES AND SPECIES)

A. D. KRIKORIAN
Department of Biochemistry and Cell Biology,
State University of New York at Stony Brook,
Stony Brook, New York,
United States of America

Abstract

Broad principles and specific points of importance in the establishment, maintenance and manipulation of suspension cultures of Musa capable of generating somatic embryos is presented against a background of published literature and the Stony Brook experience. Strategies of competent culture initiation, embryogenic culture maintenance and vulnerability of the whole of the embryogenic culture process to often unappreciated stresses are emphasized. Inadequate monitoring at the bench level of the embryogenic culture process readily leads to the kinds of problems that have earned Musa the undeserved reputation of being a recalcitrant species. Available protocols emphasize that there are alternative ways to move forward in a specific case and allow one to choose a specific route to fulfill a particular objective. The process of somatic embryogenesis is viewed here as only a small part in a multi-faceted approach to banana and plantain improvement. The cultural requirements in vitro of Musa emphasize that there is still much to learn about the developmental biology of this important group of tropicales.

1. INTRODUCTION

1.1. Distribution, importance and morphology

The genus Musa (bananas and plantains, fam. Musaceae) comprises about 25 species. The main center of diversity of these large herbaceous monocotyledons is southeast Asia but edible fruit and ornamental varieties have long been grown in the tropics and subtropics of both hemispheres.

The morphology and growth habit of these giant herbs is highly distinctive. While in the vegetative condition, the shoot apical growing point is more or less at soil level. Leaves are produced on the flanks of the meristem with very little vertical displacement of the growing tip, and leaves wrap around themselves forming a false trunk or pseudostem [1]. The lateral (leaf-opposed) buds in Musa are adventitious (cf. [1, 2, 3]). When the vegetative growing point undergoes transition to the flowering mode, the true stem tip moves upwards vertically through the hollow of the pseudostem [4, 5].

The group as a whole has been inadequately studied taxonomically. To avoid confusion, the best method available for dealing with the cultivated clones involves the use of a short-hand technique that takes into account the amount of the contribution of 'A' genome (from M. acuminata) and 'B' genome (from M. balbisiana) and some 15 characteristics [6]. A generalization is that the more of the 'A' genome, the sweeter the fruit and hence it is usable as a table fruit; the more 'B', the more starchy it is, hence it needs cooking. The better known and commercially important clones are generally parthenocarpic triploids (3n = 33) although diploids and tetraploids exist as well and have local importance.

An important taxonomic descriptor involves the persistence/deciduous nature of the male bracts, buds and axis at the terminal end of the raceme (cf. [7, 8, 9, 10]). This phenotypic change represents a significant developmental change and has major implications for fruit production [10].

1.2. Significance of somatic embryogenesis and tissue culture-related strategies

Bananas and plantains are excellent candidates for virtually all tissue culture strategies [11, 12, 13, 14]. The corms or suckers are large and inconvenient for germplasm movement, frequently
harbor pathogens and are often in short supply especially when extensive or large scale new plantings are contemplated. Germplasm maintenance in the field is expensive and space consuming, and on a commercial scale germ plasm production is usually relegated to "seed" nurseries. Breeding of the essentially seed-sterile clones of interest to plantation agriculture is fraught with many difficulties [15, 16].

A number of diseases seriously affect banana and plantain plantings throughout the world. The amount of time and money that must be invested in control measures using chemicals are beyond subsistence farmers, usually not within reach of the working class and pose financial problems even for large commercial operations. Breeding strategies to generate tolerance or resistance to important diseases such as Fusarium wilt (Panama Disease) caused by F. oxysporum f. sp. cubense (various races), Sigatoka leaf spot caused by Mycosphaerella musicola, Black Sigatoka caused by Mycosphaerella fijiensis var. difformis and to the burrowing nematode Radopholus similis and the corm weevil Cosmopolites sordidus have thus far been the main breeding objectives but these have yet to lead to commercially successful clones. The improvement programs are rendered all the more difficult because the potential to breed plantains and bananas is seriously handicapped by the high sterility of the clones of primary interest (cf. [7, 17, 18] and refs. there cited).

The reliable application of tissue, cell, somatic embryogenesis and protoplast procedures to plantains and bananas, as in the case of many other plants, is a necessary and key component in any comprehensive biotechnology plan aimed at improvement via mutation breeding and genetic transformation; it is also important for germplasm management strategies such as cryopreservation or storage and artificial seed or propagule delivery systems [12, 18, 19, 20].

1.3. Shoot tip procedures as a preliminary to suspension/embryogenesis procedures

The means to micropropagate Musa are established and have been widely adopted or adapted in various parts of the world. Technical guidelines for safe movement of germplasm have been delineated for some time (cf. [21]) and most are now aware of the problems of assuming that meristems or in vitro cultures are necessarily clean [22]. There are many possibilities of introducing virus diseases along with germ plasm if adequate precautions are not taken to see that plants are virus indexed or quarantined [18].

Various publications summarize the procedures to make primary explants and to initiate multiplying shoot cultures (cf. [11, 16, 21, 23, 24, 25, 26]). Even so, there is considerable opportunity for changes to be implemented in these methods, and refinements and attempts to understand the implications of explant selection and tissue culture management strategies for performance of propagules in the field are an ongoing activity [10]. Figure 2 in Krikorian et al [27] provides a diagrammatic representation of some of the various aseptic culture procedures for Musa and their relationships: (1) a mature plant with a ratoon or sucker. Excision of the apex from a vegetative corm (2 through 3), or alternatively a stem tip from the male floral bud (2a through 2c), yields a system where leaf-opposed and adventitious buds are generated (4 through 8). Subdivision of these multiple shoots and transfer to cytokinin-containing culture medium (semi-solid or shallow liquid) keeps the system open-ended (9). Further treatment of aseptic shoot tips in vitro with auxins can foster the development of callus, calloid, and embryogenic masses (10-13). These various-sized and variously-differentiated units may be further broken down into still smaller dimensions. These can be regenerated into somatic embryos (14a) or organogenically responsive masses (15). Each of these in turn can be reared to full-sized propagules that can be established in soil (14b, 16-17). Protoplast work can follow from any of these points but competent cell suspensions are preferable for a number of reasons [28, 29, 30].

The means whereby a shoot tip multiplication system is developed and maintained has fundamental implications for the successful establishment of morphogenetically competent suspension cultures (cf. [12, 27, 31]). M and S liquid medium [32] with 5 mg/l BAP, 100 mg/l inositol and 4 % sucrose provides an adequate starting point for initiation of multiplying shoot systems from excised stem tip or floral bud explants. Response in liquid is faster than on semi-solid medium. Rotation on a gyratory shaker is helpful but not critical. Pulsing the explant from liquid to semi-solid and back to liquid may speed up growth [23].
The methods used to carry out shoot tip culture are highly flexible but tests for bacteria and fungi must be carried out routinely and each step along the way (cf. [13, 33]). Tests for cucumber mosaic virus should be done as well (cf. e.g. [18, 34]). Other precautions will depend on the location of the operation. For instance bunchy top virus is not eliminated via meristem culture (cf. [18]) and should be tested for as appropriate [35].

Many adventitious buds are produced in vitro from cultured and subdivided shoot and floral stem tips [36, 37, 38, 39, 40] and the potential for multiplication is very high (cf. [13, 23]). Carrying out the procedures entirely in liquid medium and without agitation except the initiation of the primary explant can speed up the process even more (Krikorian and Scott, unpublished).

1.4. Callus induction

Callus culture and cell suspension culture studies using preclimacteric fruit pulp were carried out in the 1950s and 1960s (cf. [41]). Those cultures were relatively slow growing and no morphogenesis was encountered (neither was it attempted). Since then, callus induction procedures have improved but growth of somatic tissue still is not necessarily fast or routine from many Musa clones.

Multiplying shoot tip cultures provide a good starting point for callus induction since they can be demonstrably clean. Auxins like dicamba and picloram can be used but the more conventional ones like 2,4,5-T and 2,4-D are active as well. Semi-solid medium provides a somewhat better substrate in the initial stages of callus induction for research purposes since one can follow the explant more easily. The report that Gelrite is nominally pivotal to successful callus culture of banana [42] may be explained on the basis of failure to use adequately washed agar (cf. e.g. [43, 44]). Gelrite does has value, however, and can be very helpful, for instance, in detecting subtle contaminants. There can be considerable darkening in explants due to polyphenol oxidation [45] and Gelrite can help minimize this.

The most important area for obtaining explants for the production of morphogenetically competent, vigorous callus are the regions of the vegetative or floral apex and the leaf bases. The presence of multiple foci of meristematic growth at the base of the multiplying shoots is crucial to getting a sustainable culture in a timely fashion; if these are not present or induced to form, competent callus initiation and maintenance is much more difficult.

2. SOMATIC EMBRYOGENESIS

The presence of a large number of active meristematic cells in the area of the basal and primary thickening meristem of multiplying shoots in vitro make it an attractive zone from which to induce and secure vigorous and morphogenetically competent cells. This strategy is especially helpful under research laboratory conditions when one is remote from the banana growing regions and a regular supply of flowering material is not available. Excised zygotic embryos are responsive as well, and yield callus cultures and suspensions which, in turn, yield somatic embryos which develop normally although they are arguably not the choice materials since many of the edible clones do not normally produce seeds and hence any model based on zygotic embryos is flawed (cf. [46] for details on the production of somatic embryos from zygotic embryos of an ornamental diploid banana; also [47, 48, 49, 50] for work on excised zygotic embryos of a diploid M. acuminata etc). Obviously, it is preferable to initiate cultures directly from field-grown materials with proven or known qualities without the intermediate step of micropropagation if for no other reason than to save time [18, 30, 31].

Cronauer and Krikorian [51] first reported production of somatic embryos of Musa from suspension cultures of triploid clones of cooking bananas. Although their shoot apices were not well developed (cf. [52] for histological details), very small cell clusters could develop. What I call neomorphs, i.e. structures that are developmentally aberrant, and embryonal structures with varying levels of developmental and structural fidelity derived from suspensions have, since then, been routinely generated by us and others from various clones [12, 27, 31, 48, 53, 54].
Fig. 1. Photographs of some stages in the process of generating suspensions and embryogenic cultures. See text for details. A, nodular growths formed at the base of a micropropagated shoot system of an 'ABB' clone, 'Cardaba' 0.5X; section of the same, 10 X; C, 'AAB' clone 'Lady Finger' showing globular masses (lighter color interspersed between tissues which have darkened severely) produced from nodular growths similar to those in A and B, 2 X; D, view of globular masses separated from units like those shown in C, 3 X; E, partial view of a nipple flask (containing 220 ml of liquid nutrient medium) showing suspended cells, 0.33 X; F, low pH-maintained culture of embryogenic masses on a semi-solid medium. Note the uniformity and general fineness of the units comprising the culture, 4 X; magnification of the embryogenic cells shown in F after dispersion in liquid medium, 71 X; H, somatic embryos generated on semi-solid medium, 5 X.
We have initiated morphogenetically competent globules directly in liquid from bases of small shoot clusters grown on semi-solid medium. 2,4,5-T was used in our initial studies and has remained an auxin that can be used with success. We have also confirmed reports by Novak et al [31] that dicamba and picloram work as well (but picloram perhaps a bit better than dicamba to the extent that picloram cultures show less tendency to oxidize, e.g. show purple or black specks).

Somatic embryos have been generated from triploid clones using both in vitro plantlets and full size corms as explant sources [31].

We have routinely regenerated plantlets from cell suspensions of various edible clones since 1985. The procedure starts with shoot tip explants and is indirect and labor intensive. Yields have been quite acceptable (cf. Fig. 1 for representative cultured materials) and some clones give good crops of somatic embryos with good morphological fidelity. The Musa regenerants are, however, generally not of the high quality that somatic embryos obtained from other monocotyledonous systems routinely worked on in this laboratory such as daylily, or even equal to the elegant somatic embryos produced from zygotic embryos of diploid Musa.

Similarly derived somatic embryos and plantlets have been regenerated by other laboratories as well from nodular, calloid growths occurring at the base of proliferating shoots. These nodular growths are somewhat reminiscent of orchid protocorms and are said to be capable of yielding cultures which can generate somatic embryos [54, 55]. It must be cautioned that Cronauer and Krikorian [39] showed that adventive growths that grossly resemble somatic embryos can easily be mistaken for true somatic embryos. One problem is that the stem line tissue that yields the cells that can organize has been difficult to define. The precursors of the somatic embryos obtained by Sannasgala [55] and Dheda et al [54] are very much like the globular structures described by Cronauer [52] but these are perhaps better viewed as aberrant albeit morphogenetically competent, organogenetic globules, not proembryos. Novak et al. [31] reported somatic embryogenesis from suspensions generated from callus from the basal areas of shoot tips and leaf bases. Specifics as to the developmental origin of the somatic embryos still requires elucidation. Gaps exist in all the published papers particularly in those important steps between initiation of a suspension and generation of a plantlet/propagule.

For me then, the matter of routine production of somatic embryos in the edible (i.e. non-seeded), triploid clones remains in need of more work for better understanding the developmental process and needs resolution for more clones insofar as sustainability of high response once an embryogenic culture has been established.

2.1. Procedures

Most (but by no means all of the many that we have tested) clones yield elegant, vigorous shoot tip cultures, and with 2,4,5-T one can start a morphogenetically competent suspension culture. These suspensions are maintained more effectively with 2,4-D (cf. Fig. 1E), and are then followed by use of NAA to push the culture to the next level of advancement, but obtaining the right kind of growth can be and frequently is, slow especially in the beginning, slow. Selection must be carried out. The initial culture can be maintained using very finely sieved suspensions with less or greater difficulty over the long term but cultures are subject to deterioration over consecutive months of serial subculture and, unit sizes of cell clusters required prior to organized growth are often not small enough to give a highly discrete response (cf. e.g. [53, 56]). Auxins such as picloram and dicamba can also be used to initiate competent cultures. In our hands the picloram response is relatively quicker (cf. [31]).

For several years we have undertaken an alternative strategy to develop cultures which are virtually completely comprised of somatic proembryos (cf. Fig. 1F,G). These cultures retain their ability to produce very early stage somatic embryos which themselves multiply by budding. They are best maintained at the preglobular stage by use of low pH (cf. [44]). Since the "secondary" embryos, can in turn, release additional embryos, as long as the more recently generated and developed somatic embryos can be separated and subcultured, one can have a sustainable and subculturable source of somatic embryos. But the level or stage of development at which such
cultures are maintainable can vary considerably. In some instances, one can have small preembryos or globular stage embryos; in other cases, the somatic embryos can be well advanced and clearly visible to the naked eye and at a stage wherein the next level would be maturation, "germination" and further growth or sprouting. And there is, as well, the possibility of achieving and sustaining a culture with all levels of development in between. The ultimate state of a culture condition/maintenance strategy or plan depends largely at this point in time of our knowledge, on what one can get. Response seems to have a clonal origin relationship.

2.2. Strategy to obtain somatic embryos of Musa

The strategy we follow involves initiating, maintaining and manipulating cell suspensions which are (1) fairly minimally but clearly embryogenically differentiated, but, of necessity, are capable of undergoing still more advanced organized development either by adventitious routes from small callus masses, or (2) via somatic embryogenesis involving pre-proembryonic (i.e. embryogenic cell cluster) and/or proembryonic (i.e. globular stage somatic embryo) suspensions from which continued somatic embryo growth and development can derive.

Figures 1A and 1B show a representative nodular meristematic mass (which I deduce are referred to as "scalps" by some workers). When subjected to 2,4,5-T in liquid medium (Fig. 1C) these yield compact globular masses. The compact globular masses separate and can grow as units of varying size, ranging from discrete units to clusters (Fig. 1D). These units can organize and yield a nodular growth mode from which units and hence, plantlets can be proliferated. Competent cells (Fig. 1E) can be derived from the compact globular masses since they release cells more or less readily, and either adventitious or embryogenic pathways may be achieved. In the former case, shoots are generated adventitiously which must be rooted to yield plantlets; in the case of somatic embryogenesis, a process analogous to germination encountered in zygotic embryos occurs. The means, in varying degrees of detail to carry this out, may be found in Krikorian [12], Cronauer and Krikorian [24, 38, 40, 46, 52] references there cited. I emphasize here, however, that the strategy is what is crucial and there are sufficient differences among clones for us to be confident that the particulars will vary more than a little. From published reports, it is clear that more than one route of regeneration is being activated or realized -- both embryogenic and organogenic.

The work of Bakry and Rossignol [57] on "callogenesis" and production of "neoformations" (probably equivalent to the structures defined in this laboratory as calloids? -- that is, organized proliferations that initially "look" callus-like but have an epidermis in place) of various types substantiates the view that a lot of different developmental events can go on in a limited space. Adventitious shoot formation from the cultured floral axis of various so-called "determinate" plantain clones without major callus production has been routinely obtained by us (cf. [10, 37]). This indicates to us that there is a high potential for differentiation of meristems de novo ("meristemoids") from otherwise meristem-free areas.

At Stony Brook we have been able to generate competent cell masses of varying size from cells routinely sieved through number 80 sieves (177 μm pore diameter) and even number 200 sieves (74 μm pore diameter) and their ability to generate plantlets is beyond all doubt (cf. [12]). The clones most worked on are 'Cardaba' ABB (a cooking banana) and 'Lady Finger' (AAB), a dessert banana. Even so, there are many developmental questions such as understanding why it is that in some cases, small groups of cells seem to need to grow into somewhat larger masses before they organize. Is there a requirement for critical size? The greater the number of cells in a morphogenetically competent mass before competence is expressed, the greater the chance for introduction of chimeral status to the developing propagule. The implications of this for clonal stability and integrity is obvious (cf. [58] and references there cited). For me, it also follows that somatic embryos are not necessarily derived from a single cell and the suggestion by some that variation will be minimal from somatic embryos is not founded in fact. Culture dynamics and origin of somatic embryos indicates that the same kinds of risk obtain with suspension cultures and somatic embryos as with stem tips [59]. In fact, because of the numbers of somatic embryos producible with a suspension and because of the facts of how suspension cultures are initiated and maintained, the risk is even greater (cf. [60]). The value of cryopreserving such cultures is not readily apparent (cf. [61]).
Fig. 2. Schematic diagram of organized development in Musa from morphogenetically competent cell cultures. See text for details.

In our somatic embryo producing cultures there seems to be a correlation with ability to "germinate" and further grow with the level of development of the haustorium (cf. [46] and unpublished). Is there a critical nutritional- or information-processing role played by this cotyledon-like structure? Unless one can identify all the requirements for controlled germination and/or retention of dormancy or "quiescence" prior to desired use, the system is only partial and one is thereby limited to restricted and early developmental phenomena. Fig. 2 schematically emphasizes (albeit in a grossly simplified fashion) that understanding the range of responses encountered as one progresses from a micropropagated plantlet of Musa (be it diploid, triploid or tetraploid) to a vigorously dividing and morphogenetically competent culture is not a trivial task. From such dividing cells, through various avenues, one can get "units" (shown in the diagram as globules) which can give rise to various embryonal forms (including neomorphs, shown here as a "tadpole" form), organogenic masses (some discrete, others not so discrete) from callus or calloid, and also, of course, bona fide somatic embryos. In some cases all four kinds of structures are obtainable from a single culture! One can hardly call such problems as "biotechnological" ones for differential gene action is certainly at work! More effort needs to be expended in understanding all aspects of Musa somatic embryogenesis, and the greatest benefits from a practical perspective remains, for me, in the laboratory not in the field.
Acknowledgements

This paper includes work carried out at Stony Brook initially by Dr. Sandra S. Cronauer-Mitra and Dr. David L. Smith. More recently, Mrs. Mary E. Scott has played a major role in the work aimed at resolving remaining issues and helping to elevate laboratory methods to a practical level for applied use by those less experienced with *in vitro* culture methods. Support from the US Agency for International Development is acknowledged as is support for our somatic embryogenesis work on non-*Musa* species in the Stony Brook laboratory from the US National Aeronautics and Space Administration (NASA).

REFERENCES


[34] STEIN, A., LOEBENSTEIN, G. and KOENIG, R. Detection of cucumber mosaic virus and bean yellow mosaic virus in Gladiolus by enzyme-linked immunosorbent assay (ELISA). Plant Disease Reporter 63 (1979): 185-188.


Next page(s) left blank
IMPROVEMENT OF Musa THROUGH BIOTECHNOLOGY AND MUTATION BREEDING

F. J. NOVAK, R. AFZA, R. MORPURGO, M. VAN DUREN, M. SACCHI
Plant Breeding Unit, Joint FAO/IAEA Programme,
IAEA Laboratories,
Seibersdorf, Austria

A. KHATRI
Atomic Energy Agricultural Research Centre,
Tandojam, Pakistan

Abstract

Edible Musa, i.e. bananas, plantains and cooking bananas, are mostly sterile polyploids with a reproductive system that is extremely difficult to manipulate. The presently used breeding methods based on crossing of near-sterile triploids with semi-wild diploids is time consuming and laborious. The cultivation of bananas in many regions is seriously threatened by several diseases caused by pathogenic fungi (Mycosphaerella, Fusarium), bacteria (Pseudomonas), viruses (esp. bunchy top virus) and nematodes. Breeding for resistance requires new strategies and the implementation of biotechnology. The current applications of biotechnology in Musa improvement include; (1) embryo culture for hybridization, (2) shoot-tip culture in micropropagation, (3) in vitro mutation induction, (4) somatic embryogenesis and somaclonal variation, (5) in vitro screening and selection, (6) artificial seed production and (7) DNA fingerprinting applied to genomic analysis. Research has been undertaken in all these areas of Musa biotechnology. The results and ongoing projects of the Joint FAO/IAEA Programme are: (1) development of in vitro mutation systems in shoot-tip cultures, (2) development of a mutant clone with superior performance to the original Cavendish type "Grand Nain", (3) somatic embryogenesis in cell suspensions of Musa, (4) protocols for the isolation and short term culture of protoplasts, (5) the study of somaclonal variation for the genetic improvement of banana, (6) encapsulation of somatic embryos for artificial seed production, (7) selection techniques and nursery screening techniques for disease resistance breeding of Musa and, (8) protein electrophoresis and DNA fingerprinting for genomic characterization of different Musa clones. Finally, future opportunities for the utilization of tissue culture and molecular biological techniques in Musa improvement are discussed.

1. INTRODUCTION

The genetic system of Musa is extremely complicated. Sterility caused by different factors, interspecific hybridity, heterozygosity and polyploidy are common in most of the cultivated clones. The asexual nature of Musa clones is often an insurmountable barrier to cross-hybridization. The complexity of Musa genetics necessitates the use of additional technology to support conventional breeding programs. The potential of biotechnology in this crop is very high [1, 2, 3]. This report describes recent results in the development of the following in vitro systems for Musa: (i) in vitro mutation induction; (ii) cell (protoplast) culture and somatic embryogenesis; (iii) markers of genetic diversity; (iv) in vitro screening and selection for Fusarium oxysporum f. sp. cubense resistance.

2. MATERIALS AND METHODS

2.1. Materials

Clones of the following Musa species and cultivars have been used as experimental material: M. acuminata subsp. burmannica (clone IV-9), M. balbisiana, 'Pisang Mas' (AA), SH-3362 (bred diploid clone AA), Grand Nain (AAA), Cardaba (ABB).
2.2. Shoot Tip Culture

Shoot tips (2 - 5 mm in size) consisting of the meristematic dome with 2 - 4 leaf primordia were cultured on MS medium with 10 μM 6-benzylaminopurine (BAP) and 5 μM indole-3-acetic acid (IAA). The medium was solidified with gelrite (1.75 g/l). Multiple shoot formation was achieved by subculturing shoot tips on MS medium supplemented with 20 μM BAP. Shoots were elongated and rooted on MS medium with 5 μM 6-dimethylallyl-aminopurine (2iP), 1 μM indole-3-butyric acid (IBA). Detailed methods of in vitro production of banana plantlets were described by Vuylsteke [4].

2.3. Induced mutagenesis

Meristem tips, 1 - 2 mm in size, were excised from in vitro growing shoots and irradiated in a gamma cell with a 60Co source with 15 to 60 Gy at a dose rate of 8 Gy/min [5]. Uniform, longitudinally dissected meristem tips were dipped into an aqueous solution of 50 μM filter-sterilized ethyl methanesulphonate (EMS) with 2 % v/v dimethylsulphoxide (DMSO) for 3 h at the constant temperature of 28 °C [6]. The explants were then thoroughly washed with sterile water prior to culture. The nutrient media were the same as described for shoot tip culture.

2.4. Embryogenic callus and cell suspension, plant regeneration

The extreme basal parts of the leaf sheaths were cut from young leaves of in vitro meristem-derived plantlets. Corm tissue pieces without an apical meristem were cut into segments (5 x 5 mm). Both types of explants were plated on petri dishes on Schenk and Hildebrandt [7] medium supplemented with a modified mixture of Staba’s vitamins [8], 40 g/l sucrose, 40 mg/l cysteine-HCl, 30 μM dicamba and 40 g/l sucrose. The cultures were maintained in the dark at 28 °C for 4 - 6 weeks. Embryogenic calli were subcultured into liquid, hormone-free medium MS (half-strength) and incubated on shakers (120 rpm) for one week. Cell suspensions were transferred into half strength SH medium with 20 μM dicamba for long-term culture of cell suspensions (more than one year). The cell suspension maintained in the dicamba medium lost its regenerative capacity within a 2 - 3 month period. To regenerate plants, the fresh suspensions of cells and proembryogenic structures were transferred into half-strength, hormone-free MS and then plated onto solid medium composed of half-strength MS + vitamins + 40 g/l cysteine + 20 g/l sucrose, 1 g charcoal, 5 μM zeatin, 4 g/l agar and 1.75 g/l gelrite. In this double layer system, somatic embryos were converted into plants within 2 weeks. The detailed protocol is described elsewhere [8].

2.5. Encapsulation of embryos

Intact bipolar somatic embryos were transferred to a sterile 3 % (w/v) solution of sodium alginate (Sigma, medium viscosity), prepared in MS basal medium (half concentration) without calcium chloride, supplemented with Staba vitamins, 20 g/l sucrose, 100 mg/l inositol, 40 mg/l cysteine-HCl and 5 μM zeatin.

Embryos, together with the alginate solution, were then drawn up in a sterile pipette with an opening of 4 mm, and carefully dripped into a 50 μM solution of calcium chloride dehydrate on a gyratory shaker (60 rpm) at room temperature.

The beads were left in the solution for 30 minutes to allow proper hardening. Selected, totally encapsulated embryos were then transferred to the double layer medium described before.

2.6. Protoplast isolation and culture

The enzymatic mixture of Macerozyme R-10 (1 %), cellulase Onozuka R-10 (3 %) and pectinase (1 %) released a large number of palisade mesophyll cells from leaves of aseptic plants and parenchyma cells of rhizome tissue during 12 hours of incubation at 28 °C. The suspension of intact protoplasts, cell debris, starch grains and raphides was washed in CPW13M, transferred to conical tubes and after 10 minutes of centrifugation at 100 x g the protoplasts were collected from the surface of the media. The washing procedures were repeated twice to purify the protoplast
fraction. The protoplasts were cultured in the half-strength MS with 20 μM dicamba in thin layers of liquid media in petri dishes.

2.7. Protein extraction and electrophoresis

Soluble proteins were extracted from young folded leaves by using SDS buffer. Samples were homogenized and centrifuged at 13 000 rpm at 4 °C for 15 minutes. A 12 % polyaCRYlamide gel with a 4 % stacking gel was used for electrophoretic separation of proteins at 1500 V/h. The gel was stained with comassie blue.

2.8. DNA fingerprinting

Detailed procedures for DNA purification, restriction enzyme digestion and hybridization are given elsewhere [9]. The purified DNA samples extracted from freeze-dried leaf tissue were digested with restriction enzymes, Hinfl, Acul or Taql. The restricted DNA fragments were passed through an agarose gel in an electric field. The gel was denatured and dried. The DNA fragments were hybridized with single stranded DNA oligonucleotides such as CA, GTC, GATA, and hybridization was detected by autoradiography.

2.9. Fusarium (FOC) culture and bioassays of plant-fungus interactions

2.9.1. Fungal culture and filtrate production

Fusarium oxysporum f. sp. cubense was cultured on Czapek-Dox medium and transferred to potato dextrose agar for sporulation. One ml of conidial suspension containing 450 000 conidia/ml was transferred to a 500 ml Erlenmeyer flask containing 250 ml of liquid Czapek-Dox for crude filtrate production. Culture conditions were; 28 °C, continuous light for sporulation on PDA, 28 °C dark condition for production of mycelium and crude filtrate. After removal of mycelium, the liquid phase was filtered through three layers of cheesecloth, refiltered through Watmann paper No. 1 and finally filtered through a 0.22 μm Millipore membrane. Production of crude filtrate activity was assayed with a spectrophotometer following the absorbance of fusaric acid at 270 - 272 nm.

2.9.2. Bioassay of crude filtrate

Banana shoot tips cultured as already mentioned were transferred to a medium containing 3, 6, 9 and 12 % of crude filtrate obtained after 21 days and placed in the same condition as before for 3 weeks. Plant material was analyzed for various parameters including plant height, total fresh weight, shoot production and root fresh weight.

2.9.3. Plant inoculation

Rooted plantlets were rinsed to remove gelrite, the roots were trimmed, the plants were dipped into a conidial suspension (5 x 10^5 conidia/ml) for 10 minutes and then transplanted into Erlenmeyer flasks containing sterile perlite.

2.9.4. Total soluble peroxidase activity

At regular intervals infected plants were removed from the flasks and, after removal of roots and leaves, the remaining corm tissue pieces with some pseudostem tissue attached were crushed in a mortar with a phosphate extraction buffer, pH 6.8. The homogenized tissues were collected and placed in Eppendorf tubes and centrifuged for 15 min at 14 000 rpm. The supernatant was transferred to new Eppendorf tubes and immediately assayed for total peroxidase activity. Peroxidases were assayed with guaiacol as the hydrogen donor. The reaction mixtures consisted of 0.3 % (v/v) guaiacol and 15 μl of hydrogen peroxide in phosphate buffer (pH 6.8). Five μl of supernatant was added to 3 ml of reaction mixture and changes in absorbance were followed at 470 nm. The reaction was stopped after 2 min.
2.9.5. Isoelectrofocusing (IEF) of total soluble peroxidases

Isoelectrofocusing of peroxidases was performed in a horizontal gel apparatus (Pharmacia Multiphor II) at 4 °C. Ampholines (Pharmacia LKB) with a pH range of 3 - 10 were used as electrolyte carriers and prefocused for 20 minutes. Fifteen μl of sample was loaded per lane. Running conditions were 8 W fixed (mA from 33 to 10). After focusing, the gels were soaked in 100 ml of 0.1 M phosphate buffer (pH 6.8) containing 0.3 % (v/v) guaiacol and 15 μl of hydrogen peroxide. After 15 minutes of incubation the gels were analyzed for stained bands.

3. RESULTS AND DISCUSSION

The variants in \textit{MjV} \textsubscript{4} vegetative progenies of irradiated and/or chemical mutagen (EMS)-treated shoot tips included phenotypic changes in morphological (plant stature, leaf shape), physiological (sucker growth and multiplication, flowering time, fruit ripening) and agronomic (bunch quality) characters. An outstanding variant plant from the cultivar 'Grand Nain', (putative mutant 'GN6OGyA') was identified and multiplied for field testing. The mutant plant showed differences in the zymograms of soluble proteins and esterase isozymes [5] when compared to the original cv. Grand Nain. Induction of somatic mutations may substantially contribute to the broadening of the genetic variation within and among vegetatively propagated \textit{Musa} clones. The system of \textit{in vitro} mutagenesis, however, may also be important for the breeding of fertile diploids before their use for crossing. Fig. 1 schematically illustrates the biotechnology supporting the \textit{Musa} conventional breeding system proposed by Vakili [10]. Two steps of mutagenesis are proposed; (i) mutation breeding of female diploids before crossing, or (ii) mutation induction and selection among F\textsubscript{j} recombinant diploids before polyploidy induction.

The ability to induce somatic embryogenesis in economically important \textit{Musa} cultivars (AAA and ABB) has opened new approaches for somatic cell manipulation and breeding [8]. Somatic embryos may be encapsulated and used for propagation and \textit{in vitro} storage. Embryogenic suspensions represent a potentially superior source of genetic variation ('true somaclonal variation') as well as a unique unicellular system for mutation induction and selection. Embryogenic suspensions are the preferred source of protoplasts. However, attempts to induce cell wall formation and callus colony proliferation from protoplasts were unsuccessful.

Differences among 4 different clones in the soluble protein electrophoresis patterns were recognized as reliable markers for discrimination among \textit{Musa} clones of genomic group A. However, consistency in the number of polymorphic bands on electrophoretographs was dependent on the precise developmental stage of the leaves from which the protein was extracted. Young, fully folded leaves were used as standard material for sample preparation. A band of 30 000 d was present only in 'SH-3362', while a band of 32 000 d was characteristic of 'Grand Nain'. Many qualitative differences were identified among clones [11].

Specific DNA fingerprints were detected for DNA samples extracted from different genotypes. The probes and restriction enzymes used (see Material and Methods) failed to distinguish among individuals of the same clone, however, a unique DNA fingerprint was found for each clone. Further research using other probe-enzyme combinations is continuing in efforts to detect individual differences in breeding populations after crossing or mutagenesis.

Crude filtrates obtained from the FOC culture displayed toxic activity. Toxicity was dependent on the age of the \textit{Fusarium} culture and the final concentration of extract in the banana culture medium. \textit{In vitro} grown shoot tips of banana were affected by the crude filtrate and its effect was proportional to its concentration in the culture medium. No conclusive differential reaction has been obtained with respect to the known response of \textit{in vivo} susceptibility or resistance, suggesting that toxic compounds excreted by the pathogen in culture are not responsible for the different levels of resistance expressed by the clones. Alternatively the fact that the crude filtrates show a toxic activity supports the idea that toxin(s) have some role in pathogenesis, possibly during the post-infectional stage. Since the establishment of pathogenesis is a multiple step process it is likely that the different levels of resistance shown by 'Pisang Mas' and 'ISH-3362' \textit{in vivo} are due to some mechanism of early plant-pathogen interaction including wall-to-wall recognition eliciting defense processes.
In vitro mutation induction and selection for disease resistance and improved fertility before crossing

Gene(s) transformation; viral coat protein gene(s); chitinase gene(s)

Anther/microspore cultures for production of 2N homozygotes

FEMALE PARENT 2N

In vitro fertilization

MALE PARENT 2N

Intraspécific protoplast fusion - somatic recombination

DIPOIDS

F_1 recombination

Embryo culture

In vitro polyploidy induction to produce tetraploids

TETRAPOIDS (Heterozygotic)

Self pollination, backcrossing, or crossing with other tetraploids

Micropropagation of superior tetraploids

TETRAPOIDS (Selected)

Embryo culture

Micropropagation of superior triploids

TETRAPOIDS (Final selection)

DNA typing of selected clones

Fig. 1. Schematic representation of biotechnological methods supporting the conventional breeding system of Musa [10]. The techniques in dotted rectangles are not fully established in banana.
Levels of peroxidase (PRX) activity differ in the two clones. 'SH-3362' shows a ten-fold higher activity in uninoculated plants than 'Pisang Mas'. 'SH-3362' showed a sharp increase of peroxidase activity after inoculation with either Race 1 or 4. Differences have been recorded in the time of induction and in the degree of activity in the two fungal races. In fact, PRX activity in plants treated with Race 1 increases 5 days after inoculation while in plants treated with Race 4 the increase was recorded after 8 days. In 'SH-3362' treated with Race 4, the PRX activity continues to increase to reach a maximum after 11 days and then begins to decrease. In plants inoculated with Race 1 PRX starts to decrease immediately and increases once more 13 days after inoculation. These results were confirmed by IEF of infected and uninfected banana plants grown in growth chambers and showed that the increases in activity are due mainly to changes in the basic class of peroxidases. In Pisang Mas we were not able to find a similar pattern both in total activity and in IEF. Our preliminary analysis of PRX seems to indicate that this enzyme is involved in an active defence mechanism against FOC, but that in banana this mechanism is not the only one that counteracts Fusarium infection, as demonstrated by the lack of response in 'Pisang Mas' plants infected with FOC Race 1. This is not surprising, in fact the role of peroxidases has been studied in many plant-pathogen interactions and their role has not been unequivocally established in any plant-pathogen system. More investigation is needed in order to establish a role for peroxidases in the defence response to Fusarium.

4. CONCLUSION

Current applications of Musa biotechnology are very close to making a significant contribution in the breeding of new cultivars. Tissue culture techniques are being developed for the induction of heritable variation useful in Musa breeding. In vitro mutagenesis may contribute to conventional breeding programmes by extending the genetic base available for recombination. Somatic embryogenesis has been achieved in economically important cultivars. This technique is especially important for the study of "true" somaclonal variation in Musa using a unicellular system for mutation induction. The induction of disease resistance is the main objective for the use of biotechnology in Musa. Reliable selection systems for genotypes resistant to FOC may soon be available. In vitro cell selection seems to be a longer-term objective for basic research.

REFERENCES


STUDY ON MUTATION BREEDING OF BANANA, 'KLUAI KHAI'

B. SILAYOI, K. WANICHKUL, S. KEAWSOMPONG, P. SARADULDHAT
Department of Horticulture

N. SINGHABURAUDOM
Department of Pathology

Faculty of Agriculture,
Kasetsart University, Bangkok,
Thailand

Abstract

This report is composed of three parts; (1) mutation induction through tissue culture, (2) study on rooting media, (3) experiment on colchicine treatment.

Shoot tips isolated from suckers of a local banana cultivar, Kluai Khai were divided into four parts and propagated on MS medium supplemented with 15 % coconut water and 5 mg/l of BA. Subcultures were made every month until 200 plantlets were obtained. Shoots were irradiated with 0, 10, 20 or 30 Gy of gamma rays and cultured on either pH 5.6 or pH 6.1 medium. Subcultures were made until the M1V3. No proliferation was observed on pH 6.1 medium for all doses of gamma rays. On pH 5.6 medium, only the control and 10 Gy-treated explants proliferated. These were transferred to the field. However, only non-treated control plants survived.

'Kluai Khai' plantlets were irradiated with gamma rays at doses of 0, 2.5, 5.0, 7.5 or 10.0 Gy and cut longitudinally into two pieces. All were cultured on MS media supplemented with 15 % coconut water, 2 mg/l BA and 1 mg/l NAA. Subcultures were made every 4 weeks until the 8th generation (M1V8). Three media were tested for rooting : MS medium without growth regulators, MS medium with 1 mg/l NAA and MS medium with 1 mg/l NAA and 1 mg/l BA. All media were adjusted to pH 5.8. Observations were made after 4 weeks. The best rooting was achieved on MS medium without growth regulators.

To examine the effect of induced polyploidization, in vitro plantlets of 'Kluai Khai' were treated with 0, 500, 1000 or 1500 ppm of colchicine in MS medium for 24, 48 or 72 hours. The frequency of explant survival rate and adventitious bud initiation decreased with increasing concentrations of colchicine. The height, pseudostem circumference and the number of leaves produced were significantly different between treatments, but there was no difference in fruit development in any treatment. Ploidy was determined by measurement of stomatal cells and chromosome counts. Piyploid was not induced.

I. INDUCED MUTATION OF 'KLUAI KHAI' THROUGH TISSUE CULTURE

I.1. INTRODUCTION

'Kluai Khai', or 'Pisang Mas', is one of the important commercial clones of banana grown in Thailand. The fruit of 'Kluai Khai' are smaller than those of Cavendish banana, but the taste is excellent, fragrant and sweet. It is cultivated as an exotic fruit for European and Japanese markets. The demands of those markets are increasing. 'Kluai Khai' accounts for almost 90 % of the bananas exported from Thailand.

'Kluai Khai' is susceptible to leaf spot disease. This disease causes a decrease in effective leaf numbers, small bunches, undersized fingers, and low yield. As 'Kluai Khai' is grown over large areas in Thailand, the disease susceptibility of this clone is causing serious production problems.
Conventional breeding of banana is difficult due to problems associated with male and female sterility. Therefore, mutation breeding using tissue culture techniques is expected to accelerate progress in the development of leaf spot disease resistant 'Kluai Khai'.

1.2. MATERIALS AND METHODS

Suckers of cv. Kluai Khai were used in all experiments. Shoot tips were excised, divided into four parts, and propagated on Murashige and Skoog media supplemented with 15% coconut water and 5 mg/l of benzyladenine (BA). Subcultures were made every four weeks until 200 plantlets were obtained. Plantlets were irradiated with 0, 10, 20 and 30 Gy of Gamma rays. One half of the plantlets were cultured on pH 5.6 media and the remainder on pH 6.1 media. Subcultures were made to obtain $M_1V_2$ and $M_1V_3$ generations. Data and observations were recorded monthly.

1.3. RESULTS AND DISCUSSION

Multiple shoots were produced after culture of explants on MS media for 6 - 8 weeks. The number of plantlets increased at the rate of 3.3 times per month. The effects of explant culture on pH 5.6 or pH 6.1 media are presented in Tables I and II.

Table I. SURVIVAL OF IRRADIATED PLANTLETS AFTER 4 MONTHS ON pH 5.6 MEDIA

<table>
<thead>
<tr>
<th>Gamma ray treatment</th>
<th>Number of treated plantlets</th>
<th>Number of surviving plantlets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 months</td>
<td>4 months</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Tr1 (10 Gy)</td>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td>Tr2 (20 Gy)</td>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td>Tr3 (30 Gy)</td>
<td>25</td>
<td>32</td>
</tr>
</tbody>
</table>

Table II. SURVIVAL OF IRRADIATED PLANTLETS AFTER 4 MONTHS ON pH 6.1 MEDIA

<table>
<thead>
<tr>
<th>Gamma ray treatment</th>
<th>Number of treated plantlets</th>
<th>Number of surviving plantlets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 months</td>
<td>4 months</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>Tr1 (10 Gy)</td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td>Tr2 (20 Gy)</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Tr3 (30 Gy)</td>
<td>25</td>
<td>19</td>
</tr>
</tbody>
</table>

No proliferation occurred on the pH 6.1 medium. Chlorotic and necrotic symptoms were observed and the plants died. On the pH 5.6 medium, plantlets treated with 30 Gy of gamma rays did not proliferate and died eventually. Lower gamma ray doses of 10 and 20 Gy were not lethal and the explants produced 32 and 30 shoots respectively. The number of surviving plants was high on the low pH (pH 5.6) media. After 5 months, all the surviving plants were transferred to rooting media. Plantlets of Tr2 and Tr3 died. Only plantlets in the control and Tr1 groups rooted and were maintained in the greenhouse before transplanting them to the field. Eventually all of the Tr1 plantlets also died.
The results of this experiment indicated that the proper pH of the medium should be around 5.6 and that the gamma ray dose should not exceed 10 Gy.

II. EFFECT OF MEDIA ON ROOTING OF IRRADIATED 'KLUAI KHAI' PLANTLETS

II.1. INTRODUCTION

According to the results described in section I, the appropriate dose of gamma rays for 'K. Khai' was about 10 Gy, and the pH of the culture medium should be around 5.6. A factorial experiment was conducted to examine the effects of gamma ray dose and medium type.

II.2. MATERIALS AND METHODS

Five doses of gamma rays were used to treat in vitro cultured plantlets of 'K. Khai'. The doses were 0, 2.5, 5.0, 7.5, and 10 Gy. The survival rate in the M1V1 was recorded. In order to obtain chimera free material after the mutagen treatment, plantlets were subcultured to the M1V8. M1V8 plantlets were then cultured on three types of media;

1. MS without growth regulators (MS)
2. MS + 1mg/l of NAA (MS + 1NAA)
3. MS + 1mg/l of NAA + 1 mg/l of BA (MS + 1NAA + 1BA)

Thirty five replications were made for each treatment. Shoot length and numbers of roots were examined after one month.

II.3. RESULTS AND DISCUSSION

In all the treatment classes, survival was high. The M1V1 plantlets were subcultured to the M1V8 generation. The number of shoots produced in each generation is indicated in Figure 1.

The number of roots produced was also scored and these data are shown in Table III. Neither the gamma ray dose nor the medium type, affected the number of roots produced by the treated plantlets.
Table III. AVERAGE NUMBER OF ROOTS PER PLANTLET ONE MONTH AFTER SUBCULTURE (N = 35)

<table>
<thead>
<tr>
<th>Gamma Rays (Gy)</th>
<th>MS</th>
<th>MS + NAA</th>
<th>MS + 1NAA + 1BA</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2.5</td>
<td>5.0</td>
<td>7.5</td>
</tr>
<tr>
<td>MS</td>
<td>4.33</td>
<td>4.17</td>
<td>3.67</td>
<td>4.33</td>
</tr>
<tr>
<td>MS + NAA</td>
<td>6.00</td>
<td>5.33</td>
<td>4.67</td>
<td>4.83</td>
</tr>
<tr>
<td>MS + 1NAA + 1BA</td>
<td>4.83</td>
<td>3.50</td>
<td>3.33</td>
<td>4.67</td>
</tr>
</tbody>
</table>

Average     5.05ns  4.33ns  3.89ns  4.61ns  4.61ns

C.V. (%) 28.5
ns: Not significant

All M_{i}V_{a} plantlets were cultured on MS, MS +1NAA, or MS + 1NAA +1BA at pH 5.6. The length of roots was measured and these data are shown in Table IV. The gamma ray dose applied did not affect root length. MS medium without growth regulators resulted in the greatest root growth.

Table IV. AVERAGE LENGTH OF ROOTS ONE MONTH AFTER SUBCULTURE

<table>
<thead>
<tr>
<th>Gamma Rays (Gy)</th>
<th>MS</th>
<th>MS + NAA</th>
<th>MS + 1NAA + 1BA</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2.5</td>
<td>5.0</td>
<td>7.5</td>
</tr>
<tr>
<td>MS</td>
<td>9.03</td>
<td>8.02</td>
<td>8.41</td>
<td>8.40</td>
</tr>
<tr>
<td>MS + NAA</td>
<td>6.07</td>
<td>5.17</td>
<td>4.58</td>
<td>3.70</td>
</tr>
<tr>
<td>MS + 1NAA + 1BA</td>
<td>3.55</td>
<td>2.22</td>
<td>3.24</td>
<td>2.87</td>
</tr>
</tbody>
</table>

Average     6.22ns  5.14ns  5.41ns  4.99ns  5.82ns

C.V. (%) 46.05
ns: Not significant
* : Numbers followed by the same alphabetical superscript are not significantly different.

Root strength was scored by using a grading method from 1 = weak to 4 = strong. These results are presented in Table V.

Table V. ROOT STRENGTH ONE MONTH AFTER SUBCULTURE

<table>
<thead>
<tr>
<th>Gamma Rays (Gy)</th>
<th>MS</th>
<th>MS + NAA</th>
<th>MS + 1NAA + 1BA</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2.5</td>
<td>5.0</td>
<td>7.5</td>
</tr>
<tr>
<td>MS</td>
<td>3.00</td>
<td>2.67</td>
<td>2.17</td>
<td>2.83</td>
</tr>
<tr>
<td>MS + NAA</td>
<td>3.33</td>
<td>2.50</td>
<td>2.67</td>
<td>2.00</td>
</tr>
<tr>
<td>MS + 1NAA + 1BA</td>
<td>1.50</td>
<td>1.00</td>
<td>1.00</td>
<td>1.17</td>
</tr>
</tbody>
</table>

Average     2.61ns  2.06ns  1.95ns  2.00ns  2.50ns

C.V. (%) 35.24
ns: Not significant
* : Numbers followed by the same alphabetical superscript are not significantly different.
The roots of plantlets cultured on MS and MS + 1NAA were stronger than roots of plantlets on MS + 1NAA + 1BA. The gamma ray dosage did not affect root strength. Roots of plantlets grown on MS medium without growth regulators were stronger and longer than those of plantlets cultured on other media.

The doses of gamma rays used in this experiment were low, and they did not affect the survival or growth of the treated plantlets. Morphological variants such as albino plantlets, plantlets with short pseudostems, curly leaves etc., were found. However, these variants did not survive when they were transferred to soil. All of the surviving plantlets were transferred to soil for further observation.

III. EFFECT OF COLCHICINE ON Musa (AA GROUP) 'K. KHAI' IN TISSUE CULTURE

III.1. INTRODUCTION

The banana cultivar 'K. Khai', is a diploid clone with a somatic cell chromosome number of $2n = 2x = 22$. This clone is designated as a member of the AA Group. It is a parthenocarpic cultivar, as are other dessert bananas. The fruit is small and similar to those of 'Pisang Lilin'. Fruit size is about 8 - 10 cm long and 2 - 3 cm in width. It is a popular and preferred cultivar by the Thai people because of its excellent taste and aroma. It is also commercially accepted in the international market, e.g. Japanese and European markets, and a large amount of it is exported from Thailand, Malaysia and the Philippines. The export of 'K. Khai' from these three countries has increased in recent years.

The planted area of 'K. Khai' in Thailand is larger than that of Cavendish bananas, but the quality is low by export standards. Nevertheless, the taste is excellent. The small fruit, low productivity and susceptibility to leaf spot disease are the major production problems of this cultivar. Some of these might be due to the diploid nature of 'K. Khai'. Generally, triploid bananas are favoured over diploids because of their higher productivity, greater vigour and larger fruit. The greater vigour of polyploid clones may improve their resistance or tolerance to leaf spot disease under field conditions. Thus an experiment was carried out applying colchicine to meristematic tissue of 'K. Khai'.

III.2. MATERIALS AND METHOD

Different doses of colchicine were added to MS medium without growth regulators. Explants of 'K. Khai' were cultured on each MS + colchicine medium treatment for various periods of time. The following treatments were applied:

1. Control (0-0)
2. MS + 500 ppm colchicine for 24 hr. (500-24)
3. MS + 500 ppm colchicine for 48 hr. (500-48)
4. MS + 500 ppm colchicine for 72 hr. (500-72)
5. MS + 1000 ppm colchicine for 24 hr. (1000-24)
6. MS + 1000 ppm colchicine for 48 hr. (1000-48)
7. MS + 1000 ppm colchicine for 72 hr. (1000-72)
8. MS + 1500 ppm colchicine for 24 hr. (1500-24)
9. MS + 1500 ppm colchicine for 48 hr. (1500-48)
10. MS + 1500 ppm colchicine for 72 hr. (1500-72)
A completely randomized design (CRD) was used for data analysis. The following data were collected for analysis:

1. Survival rate after treatment with colchicine
2. Number of shoots produced by plantlet
3. Survival rate of plantlet after transfer to soil
4. Survival rate of plants in the field at Kamphaengsaen Campus
5. Growth rate
   - Height, pseudostem circumference, number of leaves
6. Time of shooting
   - Days to shooting, total number of leaves on day of shooting
7. Fruit
   - Bunch weight, fruits/bunch, hands/bunch
   - Fruit size (circumference, length, peel thickness)
   - Soluble solids, flesh firmness
8. Number of chromosomes
9. Leaf spot disease symptoms

III.3. RESULTS AND DISCUSSION

III.3.1. Effects of colchicine on shoot proliferation and survival rate

Data were collected one month after the colchicine treatment. Results are shown in Table VI.

<table>
<thead>
<tr>
<th>Colchicine concentration (ppm)</th>
<th>Colchicine treatment duration (h)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>0</td>
<td>3.25</td>
<td>3.25</td>
</tr>
<tr>
<td>500</td>
<td>3.25</td>
<td>3.10</td>
</tr>
<tr>
<td>1000</td>
<td>2.25</td>
<td>2.00</td>
</tr>
<tr>
<td>1500</td>
<td>2.30</td>
<td>2.10</td>
</tr>
</tbody>
</table>

Average of Colchicine treatment: 2.50<sup>a</sup> 2.40<sup>a</sup> 1.35<sup>b</sup>

C.V. (%): 72

<sup>**</sup>: Numbers followed by the same alphabetical superscript are not significantly different.

The results indicated that high concentrations of colchicine and longer periods of treatment resulted in lower shoot production. Incubation on medium containing 1,500 ppm of colchicine for 72 hours appeared to be the high end of the treatment range.
III.3.2. Survival rate of 'K. Khai' after transfer to soil

The treated plantlets were subcultured on the rooting medium in vitro before being transferred to soil. After the acclimation procedure and transfer to soil, they were grown in a planting material which consisted of mixture of soil : sand : compost manure (1 : 1 : 1) for one month. Survival data were recorded after one month (Table VII).

Table VII. SURVIVAL RATE OF 'K. KHAI' SHOOTS

<table>
<thead>
<tr>
<th>Colchicine concentration (ppm)</th>
<th>Colchicine treatment duration (h)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>0</td>
<td>80.0</td>
<td>80.0</td>
</tr>
<tr>
<td>500</td>
<td>80.0</td>
<td>80.0</td>
</tr>
<tr>
<td>1000</td>
<td>70.0</td>
<td>60.0</td>
</tr>
<tr>
<td>1500</td>
<td>70.0</td>
<td>70.0</td>
</tr>
<tr>
<td><strong>Average of Colchicine treatment</strong></td>
<td>73.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

C.V. (%) 34.9
* : Numbers followed by the same alphabetical superscript are not significantly different.

Survival rates were higher in the control treatment and lower at the higher concentrations of colchicine and with longer periods of treatment.

III.3.3. Survival rate in the field at Kamphaengsaen Campus

Three month old plantlets were transplanted to the field at the Kamphaengsaen Campus of Kasetsart University, Nakhon Pathom Province. Two hundred and fifty grams of 15-0-0 fertilizer per plant were supplied every month. On the fourth and fifth months, the formula of fertilizer used was changed to 20-20-0, but the quantity remained the same. Survival rate was examined one month after transplanting and these data are shown in Table VIII.

Table VIII. SURVIVAL RATE (%) OF 'K. KHAI' IN THE FIELD

<table>
<thead>
<tr>
<th>Colchicine concentration (ppm)</th>
<th>Colchicine treatment duration (h)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>0</td>
<td>90.0</td>
<td>90.0</td>
</tr>
<tr>
<td>500</td>
<td>90.0</td>
<td>90.0</td>
</tr>
<tr>
<td>1000</td>
<td>90.0</td>
<td>90.0</td>
</tr>
<tr>
<td>1500</td>
<td>80.0</td>
<td>70.0</td>
</tr>
<tr>
<td><strong>Average of Colchicine treatment</strong></td>
<td>86.7&lt;sup&gt;na&lt;/sup&gt;</td>
<td>83.3&lt;sup&gt;na&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

C.V. (%) 22.8
* : Numbers followed by the same alphabetical superscript are not significantly different.

The more severe treatments, i.e. higher concentrations of colchicine and longer periods of treatment, resulted in lower rates of survival.
III.3.4. Growth

The height of the pseudostem was measured monthly from the ground to the origin of the last leaf. The results are shown in Table IX. The data indicate normal growth within each treatment class, and no significant differences could be observed among the treatment classes at this stage.

Table IX. THE EFFECT OF COLCHICINE TREATMENT ON HEIGHT (cm) OF 'K. KHAI'

<table>
<thead>
<tr>
<th>Colchicine treatment</th>
<th>March</th>
<th>April</th>
<th>May</th>
<th>June</th>
<th>July</th>
<th>August</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 0</td>
<td>31.7</td>
<td>47.5</td>
<td>64.0</td>
<td>97.7</td>
<td>149.7</td>
<td>194.0</td>
</tr>
<tr>
<td>500 - 24</td>
<td>33.8</td>
<td>38.8</td>
<td>46.3</td>
<td>67.3</td>
<td>116.2</td>
<td>152.0</td>
</tr>
<tr>
<td>500 - 48</td>
<td>31.3</td>
<td>48.0</td>
<td>62.2</td>
<td>92.0</td>
<td>142.0</td>
<td>182.3</td>
</tr>
<tr>
<td>500 - 72</td>
<td>29.3</td>
<td>42.0</td>
<td>56.3</td>
<td>85.2</td>
<td>137.8</td>
<td>180.3</td>
</tr>
<tr>
<td>1000 - 24</td>
<td>34.3</td>
<td>47.3</td>
<td>54.0</td>
<td>79.0</td>
<td>123.0</td>
<td>161.3</td>
</tr>
<tr>
<td>1000 - 48</td>
<td>30.2</td>
<td>39.2</td>
<td>46.0</td>
<td>63.3</td>
<td>110.2</td>
<td>146.0</td>
</tr>
<tr>
<td>1000 - 72</td>
<td>30.5</td>
<td>42.2</td>
<td>50.8</td>
<td>76.0</td>
<td>122.5</td>
<td>158.0</td>
</tr>
<tr>
<td>1500 - 24</td>
<td>31.5</td>
<td>40.5</td>
<td>50.8</td>
<td>79.2</td>
<td>136.0</td>
<td>163.0</td>
</tr>
<tr>
<td>1500 - 48</td>
<td>32.0</td>
<td>43.7</td>
<td>55.2</td>
<td>87.8</td>
<td>140.0</td>
<td>175.7</td>
</tr>
<tr>
<td>1500 - 72</td>
<td>29.7</td>
<td>41.7</td>
<td>49.8</td>
<td>78.0</td>
<td>131.7</td>
<td>166.5</td>
</tr>
</tbody>
</table>

| F - Test | ns  | ns  | ns  | ns  | ns  | ns    |
| C.V. (%)  | 15.9| 22.0| 26.1| 27.8| 23.3| 20.3  |

Pseudostem growth was measured as the circumference in cm at a height of 15 cm from the ground. The results are shown in Table X.

Table X. THE EFFECT OF COLCHICINE TREATMENT ON PSEUDSTEM CIRCUMFERENCE (cm) OF 'K. KHAI'

<table>
<thead>
<tr>
<th>Colchicine treatment</th>
<th>March</th>
<th>April</th>
<th>May</th>
<th>June</th>
<th>July</th>
<th>August</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 0</td>
<td>10.0</td>
<td>13.3</td>
<td>17.7</td>
<td>27.3</td>
<td>37.5</td>
<td>48.3</td>
</tr>
<tr>
<td>500 - 24</td>
<td>9.1</td>
<td>12.0</td>
<td>14.7</td>
<td>20.3</td>
<td>30.5</td>
<td>44.5</td>
</tr>
<tr>
<td>500 - 48</td>
<td>9.9</td>
<td>13.7</td>
<td>18.6</td>
<td>27.3</td>
<td>38.1</td>
<td>50.7</td>
</tr>
<tr>
<td>500 - 72</td>
<td>8.7</td>
<td>12.8</td>
<td>17.0</td>
<td>25.8</td>
<td>36.6</td>
<td>49.4</td>
</tr>
<tr>
<td>1000 - 24</td>
<td>9.8</td>
<td>13.5</td>
<td>16.7</td>
<td>23.9</td>
<td>23.6</td>
<td>44.8</td>
</tr>
<tr>
<td>1000 - 48</td>
<td>9.3</td>
<td>11.6</td>
<td>15.6</td>
<td>20.5</td>
<td>28.3</td>
<td>40.1</td>
</tr>
<tr>
<td>1000 - 72</td>
<td>9.8</td>
<td>13.3</td>
<td>16.8</td>
<td>23.8</td>
<td>33.2</td>
<td>44.7</td>
</tr>
<tr>
<td>1500 - 24</td>
<td>10.3</td>
<td>12.2</td>
<td>17.2</td>
<td>24.2</td>
<td>35.3</td>
<td>44.9</td>
</tr>
<tr>
<td>1500 - 48</td>
<td>9.1</td>
<td>12.5</td>
<td>16.8</td>
<td>26.3</td>
<td>38.8</td>
<td>50.4</td>
</tr>
<tr>
<td>1500 - 72</td>
<td>8.9</td>
<td>12.1</td>
<td>15.2</td>
<td>23.6</td>
<td>33.7</td>
<td>46.0</td>
</tr>
</tbody>
</table>

| F - Test | ns  | ns  | ns  | ns  | ns  | ns    |
| C.V. (%)  | 13.0| 16.0| 23.3| 23.3| 21.1| 46.3  |

Leaf production was monitored by counting the number of leaves every month. These data are presented in Table XI.
Table XI. THE EFFECT OF COLCHICINE TREATMENT ON THE NUMBER OF MATURE LEAVES OF 'K. KHAI'

<table>
<thead>
<tr>
<th>Colchicine treatment</th>
<th>March</th>
<th>April</th>
<th>May</th>
<th>June</th>
<th>July</th>
<th>August</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 0</td>
<td>6.0</td>
<td>11.0</td>
<td>16.5</td>
<td>22.2</td>
<td>27.8</td>
<td>33.0</td>
</tr>
<tr>
<td>500 - 24</td>
<td>6.3</td>
<td>11.3</td>
<td>16.0</td>
<td>20.8</td>
<td>26.2</td>
<td>31.2</td>
</tr>
<tr>
<td>500 - 48</td>
<td>5.6</td>
<td>12.0</td>
<td>17.2</td>
<td>22.8</td>
<td>27.8</td>
<td>33.3</td>
</tr>
<tr>
<td>500 - 72</td>
<td>5.0</td>
<td>9.8</td>
<td>15.8</td>
<td>21.3</td>
<td>27.0</td>
<td>32.0</td>
</tr>
<tr>
<td>1000 - 24</td>
<td>6.2</td>
<td>11.8</td>
<td>15.7</td>
<td>21.2</td>
<td>26.5</td>
<td>31.0</td>
</tr>
<tr>
<td>1000 - 48</td>
<td>5.8</td>
<td>10.3</td>
<td>15.7</td>
<td>20.5</td>
<td>25.8</td>
<td>31.3</td>
</tr>
<tr>
<td>1000 - 72</td>
<td>5.6</td>
<td>11.3</td>
<td>16.5</td>
<td>21.7</td>
<td>26.7</td>
<td>32.0</td>
</tr>
<tr>
<td>1500 - 24</td>
<td>6.0</td>
<td>11.0</td>
<td>15.3</td>
<td>20.8</td>
<td>26.7</td>
<td>32.2</td>
</tr>
<tr>
<td>1500 - 48</td>
<td>6.5</td>
<td>11.7</td>
<td>16.3</td>
<td>21.7</td>
<td>27.7</td>
<td>33.0</td>
</tr>
<tr>
<td>1500 - 72</td>
<td>5.4</td>
<td>10.7</td>
<td>15.8</td>
<td>21.5</td>
<td>27.2</td>
<td>32.0</td>
</tr>
</tbody>
</table>

F - Test     ns   ns   ns   ns   ns   ns
C.V. (5)        14.0  6.7  7.2  5.4  4.2  3.7

The rate of leaf emergence was about 5 - 6 leaves/month or 5 - 6 days/leaf, and was stable. In contrast, the height and circumference of the pseudostem increased more rapidly during the fourth, fifth and sixth months (Figure 2).

![Figure 2. Growth of pseudostem and increase in the number of leaves.](image)

Bunch emergence occurred in September. Time to shooting was about 234.7 - 296.3 days, as shown in Table XII. The colchicine-treated plants were slower to shoot. The controls were the quickest to shoot. The number of leaves at shooting was about 43 in all treatments (Table XIII). The fruits were harvested 50 - 56 days after flowering.
### Table XII. EFFECT OF COLCHICINE TREATMENT ON AVERAGE NUMBER OF DAYS TO SHOOTING OF 'K. KHAI'

<table>
<thead>
<tr>
<th>Colchicine concentration (ppm)</th>
<th>Colchicine treatment period (h)</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>234.7</td>
<td>234.7</td>
<td>234.7</td>
<td>234.7&lt;sup&gt;b*&lt;/sup&gt;</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td>255.7</td>
<td>289.5</td>
<td>273.3</td>
<td>272.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td>263.8</td>
<td>275.7</td>
<td>276.5</td>
<td>272.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1500</td>
<td></td>
<td>260.3</td>
<td>290.8</td>
<td>296.3</td>
<td>282.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Average Trt. 259.0<sup>b</sup> 285.3<sup>a</sup> 282.0<sup>a</sup>

C.V. (%) 22.8

* : Numbers followed by the same alphabetical superscript are not significantly different.

### Table XIII. EFFECT OF COLCHICINE TREATMENT ON LEAF PRODUCTION AND BUNCH WEIGHT

<table>
<thead>
<tr>
<th>Colchicine treatment</th>
<th>Total leaves</th>
<th>No. of remaining leaf at shooting</th>
<th>Bunch weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 0</td>
<td>42.5</td>
<td>14.2</td>
<td>7 525.0</td>
</tr>
<tr>
<td>500 - 24</td>
<td>43.8</td>
<td>13.8</td>
<td>5 716.0</td>
</tr>
<tr>
<td>500 - 48</td>
<td>42.3</td>
<td>13.7</td>
<td>5 608.3</td>
</tr>
<tr>
<td>500 - 72</td>
<td>41.8</td>
<td>13.5</td>
<td>6 208.3</td>
</tr>
<tr>
<td>1000 - 24</td>
<td>42.5</td>
<td>13.2</td>
<td>6 200.6</td>
</tr>
<tr>
<td>1000 - 48</td>
<td>44.0</td>
<td>14.0</td>
<td>5 650.0</td>
</tr>
<tr>
<td>1000 - 72</td>
<td>43.2</td>
<td>12.8</td>
<td>5 558.3</td>
</tr>
<tr>
<td>1500 - 24</td>
<td>42.5</td>
<td>13.5</td>
<td>5 800.0</td>
</tr>
<tr>
<td>1500 - 48</td>
<td>44.3</td>
<td>13.7</td>
<td>5 791.7</td>
</tr>
<tr>
<td>1500 - 72</td>
<td>43.3</td>
<td>13.3</td>
<td>5 425.0</td>
</tr>
</tbody>
</table>

F - Test  ns  ns  C.V. (%) 8  6.2

The effect of colchicine treatment on bunch weight are further summarized in Table XIV. Higher concentration of colchicine resulted in lower bunch weights of 'K. Khai', but the number of fruits, number of hands per bunch, number of fruit per second hand, were not significantly different as shown in Table XV.

### Table XIV. EFFECT OF COLCHICINE TREATMENT ON AVERAGE BUNCH WEIGHT

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Duration of treatment (h)</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>Average (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>7 525.0</td>
<td>7 525.0</td>
<td>7 525.0</td>
<td>7 525.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td>5 716.7</td>
<td>6 508.3</td>
<td>6 208.3</td>
<td>6 144.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td>6 200.0</td>
<td>5 650.0</td>
<td>5 558.3</td>
<td>5 802.8&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>1500</td>
<td></td>
<td>5 800.0</td>
<td>5 791.7</td>
<td>5 425.0</td>
<td>5 672.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Average Trt. 5 905.6<sup>ns</sup> 5 983.3<sup>ns</sup> 5 730.5<sup>ns</sup>

C.V. (%) 22.8, ns : Not significant

* : Numbers followed by the same alphabetical superscript are not significantly different.
Treatment with higher concentrations of colchicine resulted in lower bunch weights of 'K. Khai'. However, the number of fruits and the number of hands per bunch were not significantly different from the control (Table XV).

Table XV. FRUITING CHARACTERISTICS OF COLCHICINE-TREATED 'K. KHAI'

<table>
<thead>
<tr>
<th>Colchicine treatment</th>
<th>No. fruit/bunch</th>
<th>No. hands/bunch</th>
<th>No. fruits/second hand</th>
<th>Weight of second hand (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 0</td>
<td>119.2</td>
<td>6.8</td>
<td>19.5</td>
<td>1 216.7</td>
</tr>
<tr>
<td>500 - 24</td>
<td>122.2</td>
<td>6.5</td>
<td>19.5</td>
<td>1 008.3</td>
</tr>
<tr>
<td>500 - 48</td>
<td>119.7</td>
<td>7.0</td>
<td>19.5</td>
<td>1 258.3</td>
</tr>
<tr>
<td>500 - 72</td>
<td>119.5</td>
<td>6.7</td>
<td>18.7</td>
<td>1 275.0</td>
</tr>
<tr>
<td>1000 - 24</td>
<td>114.5</td>
<td>6.5</td>
<td>19.8</td>
<td>1 133.3</td>
</tr>
<tr>
<td>1000 - 48</td>
<td>116.2</td>
<td>7.0</td>
<td>20.3</td>
<td>1 183.3</td>
</tr>
<tr>
<td>1000 - 72</td>
<td>135.7</td>
<td>7.2</td>
<td>19.7</td>
<td>1 300.0</td>
</tr>
<tr>
<td>1500 - 24</td>
<td>112.5</td>
<td>7.0</td>
<td>20.3</td>
<td>1 225.0</td>
</tr>
<tr>
<td>1500 - 48</td>
<td>119.7</td>
<td>6.3</td>
<td>19.7</td>
<td>1 100.0</td>
</tr>
<tr>
<td>1500 - 72</td>
<td>129.0</td>
<td>7.0</td>
<td>20.3</td>
<td>1 150.0</td>
</tr>
</tbody>
</table>

F - Test ns ns ns ns
C.V. (%) 13.6 7.8 5.6 13

The effect of colchicine treatment on fruit size was examined by measuring the fruit circumference (Table XVI), length, peel thickness and soluble solids content (Table XVII). Fruit firmness was also examined (Table XVIII).

Table XVI. EFFECT OF COLCHICINE TREATMENT DURATION AND COLCHICINE CONCENTRATION ON AVERAGE CIRCUMFERENCE (cm) OF 'K. KHAI' FRUITS

<table>
<thead>
<tr>
<th>Colchicine concentration (ppm)</th>
<th>Colchicine treatment duration (h)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>500</td>
<td>9.8</td>
<td>10.2</td>
</tr>
<tr>
<td>1000</td>
<td>10.0</td>
<td>9.3</td>
</tr>
<tr>
<td>1500</td>
<td>9.8</td>
<td>9.7</td>
</tr>
</tbody>
</table>

Average Trt. 9.9<sup>ns</sup> 9.7<sup>ns</sup> 10.0<sup>ns</sup>
C.V. (%) 5.0
ns: Not significant
* : Numbers followed by the same alphabetical superscript are not significantly different.

High concentrations of colchicine reduced the circumference of the fruit. Fruit length, peel thickness, and soluble solids content were not significantly different from the control (Table XVII).
Table XVII. EFFECT OF COLCHICINE TREATMENT ON AVERAGE PEEL
THICKNESS, LENGTH AND SOLUBLE SOLIDS CONTENTS OF
'K. KHAI' FRUIT

<table>
<thead>
<tr>
<th>Colchicine treatment</th>
<th>Peel thickness (mm)</th>
<th>Fruit length (cm)</th>
<th>Soluble solids (% Brix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 0</td>
<td>1.8</td>
<td>13.5</td>
<td>28.7</td>
</tr>
<tr>
<td>500 - 24</td>
<td>1.5</td>
<td>12.2</td>
<td>28.7</td>
</tr>
<tr>
<td>500 - 48</td>
<td>2.0</td>
<td>13.3</td>
<td>28.8</td>
</tr>
<tr>
<td>500 - 72</td>
<td>1.7</td>
<td>13.7</td>
<td>28.7</td>
</tr>
<tr>
<td>1000 - 24</td>
<td>1.5</td>
<td>12.8</td>
<td>28.8</td>
</tr>
<tr>
<td>1000 - 48</td>
<td>2.0</td>
<td>12.7</td>
<td>28.7</td>
</tr>
<tr>
<td>1000 - 72</td>
<td>2.0</td>
<td>13.3</td>
<td>28.7</td>
</tr>
<tr>
<td>1500 - 24</td>
<td>1.5</td>
<td>12.8</td>
<td>28.7</td>
</tr>
<tr>
<td>1500 - 48</td>
<td>2.0</td>
<td>13.2</td>
<td>28.8</td>
</tr>
<tr>
<td>1500 - 72</td>
<td>2.0</td>
<td>12.5</td>
<td>28.5</td>
</tr>
</tbody>
</table>

F - Test ns ns ns
C.V. (%) 7.8 6.9 1.7

Table XVIII. EFFECT OF COLCHICINE CONCENTRATION AND TREATMENT
DURATION ON FRUIT FIRMNESS OF 'K. KHAI'

<table>
<thead>
<tr>
<th>Colchicine concentration (ppm)</th>
<th>Colchicine treatment duration (h)</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.882</td>
<td>0.882</td>
<td>0.882</td>
<td>0.882*</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>0.819</td>
<td>0.920</td>
<td>0.899</td>
<td>0.879a</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.792</td>
<td>0.787</td>
<td>0.844</td>
<td>0.808b</td>
<td></td>
</tr>
<tr>
<td>1500</td>
<td>0.724</td>
<td>0.844</td>
<td>0.757</td>
<td>0.775b</td>
<td></td>
</tr>
</tbody>
</table>

Average Trt. 0.778b 0.850a 0.833a

C.V. (%) 9.3
ns : Not significant
* : Numbers followed by the same alphabetical superscript are not significantly different.

Increased concentrations of colchicine reduced flesh firmness. High concentrations of colchicine affected the development or maturation of the fruit as compared to the control. However, such effects may have resulted from damage caused by the treatment. Measurement of stomatal size, and chromosome counts revealed that the ploidy (2n = 22) was not effected by the colchicine treatment. Recently, Orizaline has been shown to be effective in inducing polyploidy (Novak, personal communication). Protocols for effective induction of polyploidy in banana should be developed, especially in combination with in vitro tissue/cell culture techniques.

III.3.5. Leaf Spot Disease Symptom

Throughout the 'K. Khai' experiments, no leaf spot disease symptoms were observed under field conditions. However, this disease did infect another variety 'K. Leb Mu Nang' which was growing near the experimental plot. The absence of disease symptoms on the 'K. Khai' plants requires investigation.
BIBLIOGRAPHY


MURASHIGE, T. and F. SKOOG A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. 15 (1962) : 473-479


Breeding of bananas and plantains has been hindered due to problems associated with sterility and vegetative propagation. Although both bananas and plantains are important as subsistence and cash crops in tropical countries they have not fully benefited from the application of conventional breeding approaches, as have many other tropical crops. Improvement of cultivars by the introduction of desirable agronomic characters through genetic manipulation, has only recently been demonstrated in these crops.

The effectiveness of mutation breeding as a means to improve vegetatively propagated crops has been demonstrated. However, in the case of *Musa*, difficulties in supplying and handling the large populations that are necessary for the application of mutation breeding techniques, have inhibited the application of mutation breeding methods.

Recent developments in biotechnology have provided new tools for the improvement of *Musa*. *In vitro* culture techniques enable the production and irradiation of large populations of somatic embryos or plantlets. Using this approach, progress in the development of improved *Musa* cultivars is expected. However, there remain some obstacles to the more wide spread and immediate application of *in vitro* mutagenesis methods for *Musa* improvement. For example, defined protocols for mutation induction and screening are needed. Problems with chimerism in regenerated mutagenized material, and ploidy level variants, must be overcome. Additional research is required on the use of cell and protoplast culture methods for mutation induction.

Development of cultivars with improved disease resistance characteristics will remain a priority. In mutation breeding programs, processes for screening of potentially useful mutants are as important as the mutation induction process itself. Appropriate screening techniques must be developed for each of the major disease organisms being investigated. Initial *in vitro* screening is preferred in order to minimize the number of plants that will require further field testing.

This Coordinated Research Program was initiated to study and solve the problems associated with the application of *in vitro* mutation breeding techniques for bananas and plantains. Biotechnology offers new and effective approaches to compliment conventional *Musa* breeding programs. Suggestions for the application of biotechnological techniques for *Musa* improvement will be presented.

There continues to be an urgent need to develop improved cultivars of bananas and plantains, since currently grown cultivars are susceptible to a number of serious diseases and insect pests. Development of improved cultivars is a difficult and time-consuming process. However, it is necessary in view of the enormous economic importance of bananas and plantains as a staple food and a currency-earning export crop. *In vitro* mutation breeding approaches will complement conventional techniques and, in combination with the novel techniques currently being developed, will speed the development of improved varieties. Close cooperation and collaboration among research groups involved with banana and plantain improvement is essential for exchange of research information and for efficient transfer of technology to developing countries.

The participants concluded the following:

1. Conventional Breeding and Mutation Breeding

   1.1. Conventional and mutation breeding programs are both important in *Musa* improvement, and can be complementary. Lack of adequate resistance to diseases appears to be the most urgent problem. However, there are a number of other breeding objectives that could be tackled using available germplasm and induced mutations.

   1.2. Embryo rescue will be an important technique for the effective utilization of *Musa* genetic resources. Embryo rescue will be used in recovering important hybrid plants from breeding
programs. Techniques to further improve the recovery of plants are necessary. In addition, techniques for embryo culture will be of increasing importance in efforts to recover cryopreserved embryos used for long-term storage of important breeding stocks and Musa germplasm.

1.3. Micropropagation from meristems and apical shoot tips continues to be an important technique for rapid multiplication of clonal materials from conventional and non-conventional breeding programs. More knowledge on mechanisms effecting the regulation of somaclonal variation is urgently needed. Variation must be minimized during clonal propagation and appropriate screening and selection techniques must be developed in order to identify somaclonal variants in micropropagated materials. Alternatively, methods to enhance somaclonal variation can be utilized in mutation breeding programs.

1.4. Anther culture techniques are being developed for the purpose of producing homozygous diploids from doubled haploids. This technique will be useful in supplying haploid materials for mutation induction. Anther culture techniques require further development. In addition, development of in vitro colchicine treatments for the production of doubled haploids, and tetraploids from improved diploid materials, is required.

1.5. For the advancement of mutation breeding technology, research on somatic embryogenesis is of continued importance. Simplification of the regeneration procedures, in order to avoid or eliminate chimerism in mutagenized regenerated callus or plants, is required. Advances in somatic embryogenic techniques, and in techniques for plant regeneration from single cells/protoplasts, are also needed.

1.6. Emphasis should be placed on the development of early in vitro screening techniques for protoplasts, single cells, somatic embryos and regenerated plantlets. Final evaluation of clones resulting from in vitro selection should be performed in countries where the proper conditions for banana and plantain cultivation exist.

1.7. Screening techniques, especially as they are applied to disease resistance, need to be developed for in vitro (mass screening), glasshouse/growth cabinet (intermediate mass screening) and field conditions (final evaluation). Clones with resistance to race 4 of Fusarium wilt are now available. Although not appropriate for commercial cultivation, these clones can be used as resistant control genotypes for comparison.

1.8. Diploid material can be used to study host/pathogen interactions, their genetic control, and also for development of resistance screening techniques. Biochemical or molecular markers that correlate with resistance genes identified in diploid materials, could be useful to help identify resistance to various diseases in mutagenized triploids.

1.9. Additional knowledge of the response of materials to the effects of various mutagens (physical and chemical) is needed. The response of various cultivars, under in vivo or in vitro conditions, must be examined. The identification of easily discernible characteristics, that reflect the effectiveness of the mutagen treatment, and that are correlated with induced mutation frequency, are needed in order to formulate appropriate mutagen doses and other treatment condition.

1.10. It is important to develop a greater understanding of the ontogenetic reactions of various types of materials (plantlets, explant tissues, callus, cell suspensions, etc.) to the mutation induction treatments, in order to be able to effectively manipulate the mutation induction process. Techniques to reduce the formation of non-chimeric plants will enhance the production of stable and useful mutants. Greater understanding of the origin of regenerated shoots (axillary vs. adventitious) is also required.

1.11. It is highly desirable to develop restriction fragment length polymorphism (RFLP), DNA amplification fingerprinting (DAF), random amplified polymorphic DNA (RAPD) and other Polymerase Chain Reaction-based molecular marker systems. These markers can be used with M. acuminata (AA) subspecies and M. balbisiana (BB) in order to identify molecular markers
correlated with morphological and agronomic traits, and disease resistance. DNA marker systems can also be useful for assessing genetic variation within pathogen species such as *Fusarium oxysporum* and the *Mycosphaerella* complex.

1.12. It also seems timely to examine cytoplasmic DNA variation using restriction endonuclease analysis of *M. acuminata* subspecies, *M. balbisiana* and the triploid cultivars in order to determine the contribution of cytoplasmic factors to agronomic characters. Due to the probable influence of *Mycosphaerella* toxin(s) on chloroplast function during disease expression, RFLP mapping of the chloroplast genome may disclose chloroplast DNA-encoded genes effecting susceptibility to this disease. Protoplast fusion, to produce cybrids carrying resistance to toxins, is an additional area for future research.

1.13. In all of the above areas, we must not lose sight of the need to produce cultivars with suitable agronomic characteristics and that are adapted to regional environments.

2. IMPROVEMENT OF RESISTANCE TO DISEASES AND PESTS OF BANANA AND PLANTAIN

2.1. One of the main objectives of mutation breeding is to obtain resistance to diseases and pests. This can be achieved by following different strategies:

(a) Changes in the degree of expression of resistance or tolerance of the host plant to the pathogen.

(b) Changes in the phenology or physiology of the host plant, such as flowering habit or speed of growth (shortening of production cycle), so as to diminish damage to individual plants.

2.2. To successfully implement these strategies a greater understanding of the pathogens, and of the host/pathogen interactions, is necessary. A better understanding of the stages and mechanisms of pathogen development in the host will facilitate the development and identification of resistant plants. A better understanding of the nature of susceptible vs. resistant host reactions is also necessary.

2.3. Research on direct interference of the host plant with the pathogen is important for development of resistant clones which involves screening and evaluation procedures:

(a) *In vitro* screening
(b) Glasshouse screening
(c) Field testing

2.4. For economic reasons, preliminary screening should be conducted as early as possible during the improvement process. Field testing is seen as the final stage of evaluation in a process of screening and selection that commenced *in vitro*.

2.5. Of the diseases and pests identified as being of importance in the genus *Musa*, the following were given highest priority:

(a) Black Sigatoka (*Mycosphaerella fijiensis* var. *difformis*) and Yellow Sigatoka (*M. musicola*)
(b) Fusarium wilt (*Fusarium oxysporum* f.sp. *cubense*)
(c) Moko (*Pseudomonas solaricearum*)
(d) Banana Bunchy Top Virus (BBTV)
(e) Nematodes

2.6. *In vitro* screening lends itself to the use of culture filtrates (toxins) of fungal and bacterial pathogens. Their function and importance in resistance/susceptible reactions requires further investigation. The use of peroxidases, and their increased production in cultivars resistant to
various fungi, also requires further study. These types of in vitro screening techniques will be of most use where resistance is based on an enzymatic or physiological reaction that operates at the cellular level. In cases where resistance is based on a morphological or structural factor this type of in vitro screening technique may have a limited application. In these latter cases, a system providing a direct contact between the host plant and the fungal pathogen (e.g. co-culture) may be required. Nevertheless, in vitro screening should be investigated as it represents a means to obtain an early evaluation, and is economical for mass screening the products from in vitro mutation breeding programs.

2.7. Screening of plantlets in a glasshouse requires the availability of standardized inoculum, reliable inoculation methods and reproducible evaluation procedures. Progress has been made to fulfill these requirements for Black Sigatoka and Yellow Sigatoka, and to partially fulfill these requirements for Fusarium wilt and Moko, although procedures should be further refined.

2.8. Preliminary experiments indicate that both environmental conditions, and the virulence of the pathogen strain, can influence the plants response to the pathogen pressure. In this regard techniques are urgently needed that enable the rapid and accurate identification, and the characterization, of pathogen isolates. This may involve the use of Vegetative Compatibility Groups (VCGs) and volatiles in the case of Fusarium wilt, or more sophisticated molecular genetic maps in the case of Black Sigatoka and Fusarium wilt.

2.9. Even when suitable laboratory and greenhouse facilities exist, testing for susceptibility using different pathogen strains must be conducted outside of banana and plantain producing countries. This restriction is necessary in order to avoid the risk of introducing new pathogens into production areas.

2.10. The ultimate evaluation of promising clones by field evaluation must be conducted in banana and plantain producing countries, preferably under several environmental conditions. Due to the resources required (land, labor, etc.), field evaluation should be limited to preselected clones. Expertise and standardized methodologies for resistance evaluation should be established in banana and plantain producing countries to guarantee reliable and reproducible ratings for pathogen response, and assessment of disease resistance. Standardization of these methods could be achieved in collaboration with the International Network for the Improvement of Banana and Plantain (INIBAP).

2.12. In some cases the physiology and phenology of the host plant may be important in conferring resistance. For instance, it has been implied that cold tolerance in Cavendish clones is correlated with resistance to Fusarium wilt, in the subtropics. By screening for cold tolerance, greater resistance to the pathogen may result. A shorter production cycle, especially the interval from shooting to harvest, would reduce host damage resulting from leaf spot diseases. The time of shedding of hermaphrodite flowers in bananas has been suggested as being related to resistance to Moko disease.

2.13. The observation of in vitro-regenerated plants and plants arising from conventional breeding programs with any of the above characters, may be important in efforts to develop resistant varieties. Researchers and breeders need to be aware of these characters when evaluating progeny from their breeding programs.

2.14. In the cases of Black Sigatoka and Fusarium wilt, susceptible and resistant clones are available as control differentials and their use is being promoted by INIBAP in an International Musa Testing Program. They may be used as control materials for the screening of mutagenized populations, although they may be valid as control plants only for the mechanism of resistance unique to them. Screening methods should be capable of detecting new resistance mechanisms.

2.15. No natural resistance to BBTV has been observed in the genus Musa, therefore appropriate screening and selection techniques still need to be developed for this disease. Control of BBTV currently depends on the development of suitable indexing procedures and the
movement of only certified clean planting material. In addition to mutation breeding for resistance, there may be an application for the use of plant transformation techniques using virus coat protein gene(s) and/or inoculation of material using attenuated strains of virus. It is highly desirable that these research programs continue.

3. INTERNATIONAL CO-OPERATION AND CO-ORDINATION

3.1. The dimensions of the task ahead require considerable international cooperation, due to the breadth of the knowledge of the materials and objectives involved. Cooperation with the International Network for the Improvement of Bananas and Plantains (INIBAP) is desirable as is collaboration with the international agricultural research centers such as the International Institute of Tropical Agriculture (IITA) or Centro Agronomico Tropical de Investigacion y Ensenanza (CATIE).

3.2. Various regional and national programmes are supported through FAO, IAEA, UNDP and various other donor agencies. Strong national programmes form a solid foundation for international coordination. Strengthening of national programmes should therefore be a high priority in international programmes.

3.3. The Agriculture Laboratory of IAEA's Seibersdorf Laboratories near Vienna, has emphasis on the development and application of technology related to the use of mutagenesis for plant improvement, and on transferring such technology to breeders in member states. Particular attention is currently paid to bananas and plantains where, due to the difficulties associated with conventional breeding approaches, mutation breeding may play a crucial role in improvement efforts. Bananas and plantain breeders should be encouraged to utilize the various services offered by the Laboratory, particularly its training programme and for assistance in mutagen treatments.
PARTICIPANTS IN THE CO-ORDINATED RESEARCH PROGRAMME

Gonzales, E.S. Universidad Nacional,
Escuela de Ciencias Agrarias,
Apartado Postal 86,
Heredia, Costa Rica

Jarret, R.L. USDA-ARS,
1109 Experiment Street,
Griffin, Georgia, 30223-1797, United States of America

Krikorian, A.D. State University of New York at Stony Brook,
Department of Biochemistry,
Stony Brook, New York 11794-5215,
United States of America

Mendes, B.M.J. Universidad de Sao Paulo,
Campus de Piracicaba,
Avenida Centenario 303,
Caixa Postal 96, CEP 13.400 Piracicaba, SP, Brazil

Perea-Dallos, M. Biology Department,
Universidad Nacional de Colombia,
Bogota, Colombia

Siddiqui, S.H. Plant Genetics Division,
Atomic Energy Agricultural Research Centre,
Tandojam Sind, Pakistan

Silayoi, B. Department of Horticulture,
Kasetsart University,
Bangkhen, Bangkok, Thailand

Smith, M. Maroochy Horticultural Research Station,
Queensland Department of Primary Industries,
P.O.Box 5083, S.C.M.C.,
Nambour, Q 4560, Australia

Valerin Aguilar, A.T. Universidad Nacional,
Escuela de Ciencias Agrarias,
Apartado Postal 86,
Heredia, Costa Rica

Yang, P. Beijing Institute of Nuclear Engineering,
P.O.Box 840,
Beijing, 100840, China

Scientific Secretary

Amano, E. Plant Breeding and Genetics Section,
Joint FAO/IAEA Division, IAEA,
Wagramerstrasse 5, P.O. Box 100,
A-1400, Vienna, Austria
Novak, F. Plant Breeding Unit,
Joint FAO/IAEA Programme,
IAEA Laboratories, Seibersdorf, Austria

Observers at the second Research Co-ordination Meeting, San Jose, Costa Rica

Dercksen, P.M. FAO Representative, Costa Rica
Guendel, F. Universidad Nacional, Costa Rica
Rivera Coto, G. Escuela de Ciencias Agrarias, Universidad Nacional, Costa Rica
Salazar Androvetto, R.A. Universidad Nacional, Costa Rica
QUESTIONNAIRE ON IAEA-TECDOCS

It would greatly assist the International Atomic Energy Agency in its analysis of the effectiveness of its Technical Document programme if you could kindly answer the following questions and return the form to the address shown below. Your co-operation is greatly appreciated.

Title: In vitro mutation breeding of bananas and plantains
Number: IAEA-TECDOC-800

1. How did you obtain this TECDOC?

[ ] From the IAEA:
  [ ] At own request
  [ ] Without request
  [ ] As participant at an IAEA meeting
[ ] From a professional colleague
[ ] From library

2. How do you rate the content of the TECDOC?

[ ] Useful, includes information not found elsewhere
[ ] Useful as a survey of the subject area
[ ] Useful for reference
[ ] Useful because of its international character
[ ] Useful for training or study purposes
[ ] Not very useful. If not, why not?

3. How do you become aware of the TECDOCs available from the IAEA?

[ ] From references in:
  [ ] IAEA publications
  [ ] Other publications
  [ ] From IAEA meetings
  [ ] From IAEA newsletters
  [ ] By other means (please specify)
[ ] If you find it difficult to obtain information on TECDOCs please tick this box

4. Do you make use of IAEA-TECDOCs?

[ ] Frequently
[ ] Occasionally
[ ] Rarely

5. Please state the institute (or country) in which you are working:

Please return to: R.F. Kelleher
Head, Publishing Section
International Atomic Energy Agency
P.O. Box 100
Wagramerstrasse 5
A-1400 Vienna, Austria