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MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18
FROM: AID/SCI, Victoria Ose *VO*
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 3, F-51
Final Report

Attachment

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Final Project Report and Recommendations

Meristem Culture for Multiplication of
Disease Tolerant Clones of Plantains and Cooking Bananas

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Summary of Completed Project and Recommendations

The project sought to assess the feasibility of developing and using meristem culture and related methods for use in screening, selection, production and multiplication of cooking bananas and plantains that are resistant or tolerant to Black Sigatoka and other diseases. We have established that vegetative apices can be isolated from shoots of any size although large shoots are easier to work with. While they are easier to work with, it is more risky to do so since cryptic contaminants cannot be easily eliminated in large shoot tips. Small apices, on the other hand, down to the size of discrete meristems are of great use in producing disinfested and/or specific-pathogen-free plants. Our work and interaction has benefitted the establishment at CATIE, Tropical Agricultural Research and Training Center, Turrialba, Costa Rica of an ongoing Musa tissue culture program under the direction of Dr. Ludwig Muller, Plant Physiologist. Also, it has enabled a plant tissue culture laboratory to become operational in Panama City at IDIAP under the direction of Susana Pons. Work on Musa is in progress there as well. The laboratory is physically located at the University of Panama. Other laboratories have likewise benefitted throughout Latin America as a result of invited participation in courses, seminars, and congresses. While plantain and banana tissue culture work has been emphasized in these places, other systems of interest to the people in question have been discussed as well.

Project-Related Tasks accomplished include:

- 1) Aquisition and preparation of a data base from laboratory and field studies (limited as they are) for capability of assessing the feasibility of using excised shoot tip, tissue, cell and protoplast culture for the rapid multiplication, storage and distribution and

improvement and conservation of cooking bananas (plantains) and bananas.

- 2) Definition of key areas of basic and applied research necessary for full implementation of cooperative research efforts in Central and Latin American and other LDC's.
- 3) Collection and preparation of select and superior Musa germ plasm for return to and further work in, the Stony Brook laboratory environment. This has involved explanation of shoot tip material and floral male bud meristems under aseptic conditions from carefully selected disease tolerant or resistant plants. This has been done both in Costa Rica at CATIE and Panama, I.D.I.A.P.
- 4) Participation in "on the spot training" and interaction with Dr. Ludwig Muller and his student-investigators at CATIE and in Panama at I.D.I.A.P. with Susana Pons and her laboratory personnel. This has involved instructing them in the methodology as we have developed and used it and discussing with them, details and problems for various laboratory tissue culture investigations on plantains and bananas. Other courses in Latin America have been participated in as a result of invitations from contacts gained through the courses e.g. Venezuela, Colombia, Chile, Argentina. The same is true for India, Indonesia, Malaysia, Philippines.
- 5) Initiation and completion of elucidation of detailed research plans and proposals to address comprehensively and systematically the goals and needs in several different and contrasting settings--CATIE and I.D.I.A.P. (Panama)
- 6) Examination and discussion of the development and implementation of tissue culture practices complementary in both the short and long

term context to those used in conventional garden, field or plantation practices.

- 7) Development of strategies to further extend and refine methods already developed at Stony Brook for other plants to cooking bananas and plantains thought or known to have tolerance to "Black Sigatoka".
- 8) Over two dozen substantive and important publications have resulted from the activities. Pre-prints and reprints and personal briefings in person and by telephone have routinely been made available to collaborators and all those interested in a very wide geographic area ranging from Puerto Rico and the U.S. Virgin Islands to Australia.

The responsiveness of Musa apices to in vitro multiplication procedures is not limited to vegetative apices. Floral apices, when isolated and cultured in an analogous procedure, will respond by yielding vegetative buds. These floral primordia will reorganize to yield a vegetative system, give rise to small vegetative shoot clusters which are similar, but may not be identical, to those from vegetative apices. The shoots can be rooted easily. Proembryonal globules, capable of giving rise to somatic embryos, can be produced from multiplying shoot systems in liquid media using 2,4-D and 2,4,5-T. Somatic embryos can be generated from seeded diploids with great success and plants can be recovered in large numbers. Certain triploids seem to be responsive as well. The diploid system which has been under development can thus provide a much-needed model for the study of somatic embryo formation in Musa. It is more important, however, that the important triploids be made to respond as well.

The shoot tip methods developed are impressive when one considers that a single isolate of the vegetative shoot growing point of a banana or plantain can yield, in theory at least, several million plants in a year. It is also impressive if one considers that the floral meristem (the male bud growing zone or point) is equally responsive and can also be used as a source of explant material - at least for test multiplication.

There is, without doubt, every reason to believe that the tissue culture multiplication methods can play a major role in enabling reliable

- rapid expansion and availability of new clones and hybrids
- production of disease-free or specific-pathogen-free planting material for new areas
- germ plasm shipment and exchange
- production of plants for research purposes including tolerance testing against challenge of disease, insects, nematodes; use in nutrition and other physiological studies.

The shoot tip and meristem methods also hold promise for genetic conservation of germ plasm and gene bank maintenance.

The shoot tip and meristem methods may also hold the key for production of new, improved bananas and plantains via mutation breeding or related procedures.

But we need to know more of the basic science and practical problems involved in implementation.

For instance, we still do not know with any high degree of confidence the extent to which one can infallibly rely on the tissue culture methodology from the perspective of the "clonal fidelity" of Musa plants produced from meristem or shoot tip culture. Field testing of our materials has been generally limited and only relatively recently has information begun to

emerge from relatively small scale plantings and trials. It is fair to say that most field trials have been small except for a few notable exceptions involving dessert (export) bananas. Our cooking banana material seems to be highly stable (see also Muller, 1987 final report). The shortcoming of reports that some plantings, particularly dessert bananas, have been erratic has caused confusion since one does not know precisely what was "tissue cultured," and more importantly how it was carried out. I have attempted over several years without much success to get large scale plantings of precisely grown, aseptic cultured-generated plants field tested. The arguments against large field trials have included the cost, and more significantly, the view has been repeatedly expressed (to my great frustration I might add) that nothing "new" could emerge since the shoot tip or meristem culture techniques were now "well established" and "routine". Nothing is further from the truth! I suspected from the outset of our own work undertaken some years ago that the outcome vis a vis stability was concerned might depend on what was done and how it was done. There has been information for quite a few years that plantlets of many species produced via "tissue culture", even organized shoot tip culture, are not necessarily true to type. The changes encountered may, in some instances, be rather extensive. Additionally, they seem to vary according to the plant and the exact procedures used. There is very little good, basic information on any plant of the causes of the variation. The changes are frequently "lumped" under the all-inclusive and generally uninformative designation of "somaclonal variation."

In general, it is supposed that the more organized the initial starting explant, the less the variation. The less organized the cultured starting material, the more the variation. On this view, meristems and shoot tips

would produce less variation, and callus, cell suspension or protoplast procedures would generate the most varied plantlets. Even so, substantial variation can be and has been encountered in some shoot tip generated materials in several plant species other than Musa. Since Musa in particular is a genus that has shown considerable potential for variation and mutation, it seemed reasonable to me long ago that useful variation could emerge from tissue culture. Indeed, as many or more examples of useful variants being grown on a large scale could be cited for banana and plantains as perhaps any other vegetatively cultivated plant.

There is every reason to believe that the challenge and opportunity exists now to undertake careful studies to ascertain whether we can control clonal stability of important genotypes of Musa in vitro for use in those cases where stability is a sine qua non and/or whether useful and agronomically significant variants can be produced by aseptic meristem or shoot tip culture methods. The encouraging but still very preliminary results obtained by S.C. Hwang and his colleagues in Taiwan, at the Banana Research Institute, Pingtung, with 'Cavendish' clones is a case in point on behalf of the potential for useful variant selection. By observing field performance of shoot tip culture-generated plants, they encountered plants with varying degrees of tolerance to Panama Disease or Fusarium wilt race 4. This is but one example of the possibilities for generating disease-tolerant plants (Proceedings of ACORBAT Meetings, San Jose, Costa Rica, 1985, still in press). But even here we immediately run into a problem. There is a published report that the shoot tip multiplication system as used in Taiwan is nominally capable of producing high level clonal stability in Cavendish clones (Hwang, Chen, Lin and Lin, 1984). When I first heard from Dr. Harry Stover of the somaclonal variation encountered in Taiwan as to tolerance

against Race 4 Fusarium wilt, I had trouble reconciling this view with the contents of the above cited paper from Taiwan reporting stability of type. Even now, from my perspective, this discrepancy has not been adequately resolved.

At the third Interantional Conference on Plantains and Other Cooking Bananas held 28-31 May 1985 in Abidjan, Ivory Coast, Dr. Stover presented some slides of that work and at the ACORBAT Meetings in San Jose, Costa Rica later that summer, more detailed results were presented by Dr. Hwang. It was unclear to me, or to Dr. Hwang when he was asked what was the cause of the variation. Dr. Hwang had no precise information on the tissue culture methods used in that work. I have read the laboratory reports from Taiwan and they are not clear. We are in contact with the Taiwan laboratory. In fact they have expressed a desire to spend time at Stony Brook.

More recently, the frustrations and disappointments experienced over the poor performance from a clonal perspective of substantial 'Grand Nain' banana plantings in Jamaica using tissue culture-derived plants raises additional questions. Depite repeated statements from Oglesby Nurseries in Hollywood, Florida (the contract suppliers of tissue culture generated plantlets on behalf of United Brands) that the materials they produced were strictly clonal and free of specific pathogens, I was sceptical of this and said so. At a tissue culture meeting at the U.S. Department of Agriculture in Beltsville, Maryland October 20-22, 1985 I specifically and publicly asked the senior Oglesby about clonal stability of the Jamaica banana plantings at the end of an illustrated presentation he made on the topic. Apparently the "off-types" have turned out to be some 20% (copy of a letter to me from Dr. H. Stover). Certainly this would appear to be a set-back in the use of

tissue culture to produce clonal plantings (See however, Oglesby and Griffis, 1986).

All the above indicates that we must continue to appraise, critically, and scientifically, the specifics in great detail of the tissue culture from shoot tips or meristems of bananas and plantains. If research work that we feel is important to pursue is to go forward, and if the proper scientific base is to be firmly established, this must be done. Similarly, whether one likes it or not, we must face up to the fact that valuable time has been lost due to poor judgement on the part of some. My sincere hope is that we can put all that behind us and now do what needs to be done to capitalize upon the potential to produce significantly improved plants.

Final comment

The single biggest frustration for me has been the recognition that so much needs to be done and the constraints are many. The constraints are financial and this is real but the most significant constraint is that an educational base on which one can really build and grow is lacking in virtually all the places I have visited and worked in.

The idea, or perhaps more accurately the notion, that plant tissue culture activities represent a risk-free technology--ripe for exploitation--without regard to fundamental science causes much of the difficulty in making the kind of real progress one aspires to. Unless more is done to teach basic plant biology from the modern perspective of developmental biology, little of real and lasting value will be achieved. Transfer of technology will, in the meantime, be the best approach but since progress is constantly being made in research, one cannot spend the rest of one's life "transferring technology".

Our activities have shown that the grant has paid off handsomely. Much has been learned and taught.

Perhaps the most satisfying aspect of all has been that Susana Pons has enrolled in a Ph.D. program (in France because their residency requirements are virtually non-existent). I have been asked to be a major advisor to her thesis work.

Since Susana has two children, this arrangement is going to be the one most workable. She can remain in Panama and spend time in France and Stony Brook as conditions or opportunities permit.

A mechanism must be found to allow all this to happen.

I am also pleased to report that Sandra Cronauer (now Cronauer-Mitra) completed her Doctoral Dissertation in June 1986 on "In Vitro Growth Responses of Musa". Copies of this thesis have been presented to collaborating institutions.

Finally, I am pleased that Dr. Muller will, with Dr. Reuveni of Israel, pursue additional research on plantains and bananas. Dr. Reuveni and I have known each other for years. Even as that work progresses, it is my hope to pursue cell and protoplast culture techniques so that a fuller complement of strategies can be implemented in terms of dealing with Musa and its problems and improvement etc.

Project Related Publications

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Background Information and Results

I believed before my initial proposal to A.I.D. was submitted, and believe even more firmly now, after our work, (see project related publications, page 12) that there is considerable potential for safeguarding the world's plantain and cooking banana resources, and for plantain and cooking banana improvement by the use of modern meristem, tissue, cell and protoplast culture techniques. These range from methods for rapid, clonal multiplication of specific pathogen or disease-free material from shoot tip or meristem cultures on the one hand, and the generating of useful genetic variation on the other hand. I will again emphasize however, that I still do not see the aseptic culture techniques as replacing more traditional methods and approaches. Instead, one sees them as supplementing them and being used in combination. At best, they can "go hand in glove". Although Champion (1963), Stutzky et al (1964), deLanghe (1969), Menendez (1973), Broertjes and van Harten (1978) and others apparently early envisioned mutagenesis of suckers as a method of banana-plantain improvement, the means whereby this vision could be even attempted has only relatively recently come to light. At the same time, bananas and plantains provide very interesting systems on which to work so as to gain answers to fundamental questions in plant biology--especially cell biology and development. Because plantains and bananas are monocotyledons, they do not generally and reliably yield to those techniques commonly used to initiate, produce and maintain cell suspension cultures from dicotyledons. Also because almost all of the edible clones of Musa do not produce seeds, one generally does not have seeds or seedlings to experiment with from the perspective of a possibly valuable starting material for tissue culture studies. Meristem culture (more

accurately shoot tip culture) has thus far provided the most readily prompt strategy to start cultures. This is what we have done.

There is now a well established and an increasing body of published literature on the shoot tip and apical meristem culture of bananas and plantains. The first and very preliminary study was published in 1972 (in Chinese). There was no particular pre-occupation in that publication for details, and dissemination of the specifics needed to repeat the work were lacking. We therefore had to start the whole business de novo and have now refined the methods and quantified them. We have also seen to it that detailed protocols for shoot tip and meristem culture are now available for all to use. This should go far towards permitting investigators and technicians to achieve what I believe to be "state of the art" capability (see Krikorian and Cronauer, 1984a; Cronauer and Krikorian, 1984a and b;1985).

The vast majority of effort insofar as stem tip culture is concerned is, at least on a world wide basis, perhaps being placed on AAA or dessert banana clones. Work in the Stony Brook laboratory on both AAA bananas and AAB and ABB plantain and cooking banana clones has shown that there is considerable variation in response in culture due to genomic differences in sucker production. Our findings with the cooking bananas 'Pelipita', 'Cardaba' ['Saba' alliance], 'Gana Auf', and the plantain 'Harton' indicates that the multiplication rates vary substantially (Cronauer and Krikorian, 1984a). Fortunately, most responses are more than adequate in terms of material producible in aseptic culture. Even here, however, it is still not clear which of the three kinds of buds known to ostensibly exist in plantains and bananas, are being formed in vitro and which culture conditions foster one

kind of bud versus another. This has significance for the potential outcome vis-a-vis clonal and phenotypic stability (Cronauer and Krikorian, in press).

A potentially important discovery in this regard at Stony Brook has been that we now know that the same kind of strict meristem system achievable with vegetative apices can be initiated from floral apices (Cronauer and Krikorian, 1985). This work has shown that the apical dome of flowering Musa seems not to be irreversibly determined and that it can be stimulated to embark upon a growth mode that is vegetative rather than floral. It has given us a valuable procedure whereby we can manipulate, relatively easily, the size of the initial apical explant. The vegetative apex is much more difficult to work with since it is buried deep within the corm and the apical dome does not, therefore, readily lend itself to removal, with any degree of confidence, along with controlled amounts of subjacent or lateral meristematic tissue. In contrast, the meristematic region of the floral apex (male bud) is more prominent and protrudes in such a way that it can be excised precisely (Cronauer and Krikorian, 1985). This may well prove practically useful in achieving specific pathogen-free material whether from viruses, viroids, mycoplasmas etc. It also provides the attractive option of multiplying a clonal population without upsetting the normal sucker production sequence of a new clone which is available only in limited supply.

The means to establish whether bacteria and fungi-free materials have been produced in a tissue culture setting are now more or less straightforward to implement. Testing by specific nutrient broth for bacteria and fungi has been followed routinely in this laboratory but has not, surprisingly, been routinely practiced even by responsible tissue culturists concerned with mass propagation, much less those in the commercial sector. Provided precautions are taken and monitoring is rigorously

exercised, contamination problems can be handled. Our work has shown, though, that if one is not meticulous, contamination which is not generally visible can enter into the multiplication system. No attempt will be made here to deal with the challenge posed by the situation of ascertaining incontestibly that materials are specific virus or viroid free.

I presented a scheme some time back which need only be referred to here (cf. Figure 2 of Krikorian and Cronauer, 1984b) that raises the very real possibility of shoot or meristem culture procedures being used to generate or select spontaneous mutants or culture associated variants of Musa clones. To do this one would need to expose very large populations of regenerated plants to pathogen challenge. It would be a major (but by no means impossible) undertaking, but there is no doubt that it would be a worthwhile effort. One of our problems at Stony Brook has been the inability to obtain funds for field testing large populations of aseptically generated plantlets in locations known to be troubled by pathogens. Smaller populations have been field tested, however, and show interesting potential. Even so, there is no doubt in my mind that the chances of selecting potentially useful spontaneous induced mutations via cell culture techniques could even more greatly improve retrieving disease-tolerant lines of plantains and bananas, since plantlets could, in theory at least, arise from single cells in virtually unlimited numbers. The most challenging and rewarding work involving tissue culture techniques will in my view, entail initiation of suspensions (in contrast to meristem, shoot tip, tissue or callus cultures) that have a capacity for production of plantlets either by initiation of shoot and root growing points adventitiously on a callus derived from cells, or by the formation of adventive or somatic embryos. The latter would be highly desirable from both the view point of either micropropagation,

somaclonal variant production or "mutation breeding" (see Krikorian, 1982; Cronauer and Krikorian, in press).

If one subscribes, as I do, to the view that a basic cell biology approach holds great promise for a long term solution to various physiological or pathogenesis problems of plantains and bananas, an early priority must still be to develop better cell methods. There is much evidence that can be cited to demonstrate conclusively that tissue culture methods in the broadest sense of the word are capable of producing variations and mutations. I believe that we will be able to apply tissue culture to select or generate new variations of plantains-bananas which are tolerant to 'Panama Wilt Disease' and perhaps 'Black' and 'Yellow Sigatoka'. Efficient capability for screening and selection of disease tolerance or resistance is impossible, however, without the effective and full development of a wide range of aseptic cell culture methods.

Induction, at will, however, of totipotent cell suspensions of any plant (much less plantains or bananas) is generally appreciated as being very difficult to achieve. It is not simply a matter of the extension of existing "technology"; it is not a "routine matter." It is a basic research problem. In this project we have also directed some efforts to probing the potential for working with cell suspensions of various Musa clones. We have also done some studies with protoplasts (see Cronauer and Krikorian, 1987). The results with each of these systems, which we need not elaborate here (see publications appended), have underscored the generally recalcitrant nature of the genus. The progress with cell suspension culture has been slow when candidly evaluated in terms of real practical potential. We have, however, been able to induce from the 'Pelipita', 'Cardaba', and 'Harton' the formation of structures substantially like somatic embryos. These structures

can form roots but the shoot development is poor and generally arrested (Cronauer and Krikorian, 1983). More recent efforts to induce new growth forms or to initiate different modes of growth from aseptically cultured materials have disclosed scientifically interesting results but the demonstration that these will have any implications for immediate practical goals await further work (Krikorian et al, unpublished; Cronauer and Krikorian, unpublished.)

Excellent results have been obtained using excised embryos of the diploid, seeded banana Musa ornata Ruxb. as starting materials for somatic embryo production. Although this in-edible banana is of interest only as an ornamental, it has demonstrated to us that somatic embryos can be obtained in profusion. Moreover, these somatic embryos can be germinated and reared in soil to maturity (Cronauer and Krikorian, in press).

A main future priority must be to develop further and to render new cell culture systems for Musa as reliable as possible (see Fig. 1). This means continuing to probe the systems carefully and systematically. A considerable store of knowledge has been accumulated by us as a result of the project. The clones on which studies should now concentrate would include those suspected or known to be good breeding parents, as well as those like the 'Horn Plantain' which is susceptible to Black Sigatoka and which is highly prized as a good food source. Similarly, 'Cardaba' and 'Pelipita' are examples of Sigatoka-tolerant cooking bananas which have many good qualities but could be vastly more useful if change in other less appreciated characteristics could be brought about. The rationale here is that it would be best to work with clones with good qualities that would require relatively little, but nonetheless critical modification to render them useful (Shepard, 1980; Gottschalk and Wolff, 1983). However, as wide a range of clonal

materials or biotypes as necessary should be investigated based on the knowledge of the potential they would have for disclosing principles of regeneration in culture, of stability and instability of genotype, or of conferring tolerance or resistance to various clones. Basic, scientific information must continue to be obtained on how to use the tool of tissue culture for the type of problems faced by those seeking to improve plantains, cooking and dessert bananas. This will not be achieved overnight. The new technologies still offer no immediate and controlled solutions. Even so, the progress has been encouraging and the impetus of research must not slide.

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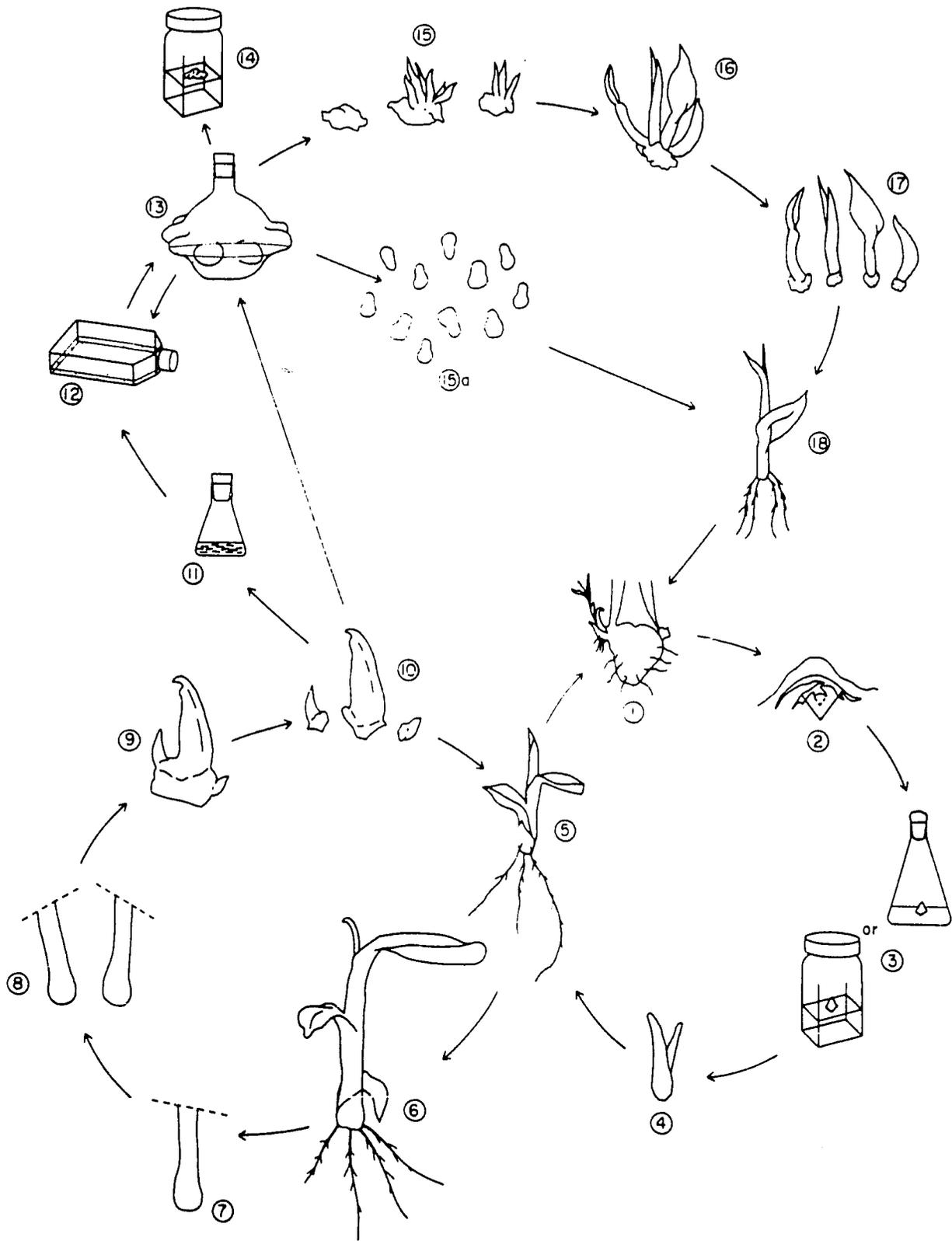
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Fig. 1. Diagrammatic representations of the procedures followed in the establishment of aseptic shoot apex or meristem cultures (1 through 3) and the formation of rooted plantlets (4 and 5). Multiple branching cultures can also be produced (6 through 10). Somaclonal variation (spontaneous change or mutation) or challenge with mutagens have promise to produce tolerant variants but work with free totipotent cells or protoplasts, theoretically at least, would provide the greatest chance for controlled, successful changes being introduced. Investigation of the isolation and regeneration of protoplasts and cells from our cultured material (11 and 12) should continue. This scheme includes developing procedures for the regeneration of protoplasts to produce suspension cultures, callus, and plantlets (13 through 18) or ideally, somatic embryos and plantlets (15a and 18).



3 F-51

Appendix: Publications and pre-prints. Arranged such that uppermost is most recent; lowermost is oldest.

Note: Full citation available from listing of project related publications. All those provided here have either been published or are in press. In latter case the preprints have been circulated to those concerned.

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Plant Regeneration via Somatic Embryogenesis in the Seeded
Diploid Banana Musa ornata Roxb.

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ABSTRACT

Somatic embryos of a seeded diploid ornamental banana (Musa ornata Roxb.) were obtained from zygotic embryos cultured on semi-solid Murashige and Skoog (1962) medium with the auxin 2,4-D (0.5, 1, 2 mg/l) and 5% CW. A series of subsequent media changes including Schenk and Hildebrandt (1972) salts led to embryo germination, growth and plantlet production. The remarkable phenotypic fidelity of somatic embryos to that of zygotic embryos and the presence of a haustorium-like outgrowth on the somatic embryos is described.

INTRODUCTION

In the past several years, plant regeneration via somatic embryogenesis has been reported for an increasing number of monocotyledonous plant species. Frequently, these cultures are initiated from excised zygotic embryos (Botti and Vasil, 1983; Vasil, et al., 1985; Hakman and Fowke, 1987) but other plant parts, most notably young leaves, have also been used (Lu and Vasil, 1981; Yeh and Chang, 1986; Szabados, et al., 1987)

The vast majority of work on Musa clones has concentrated on the production of multiplying shoot systems from cultured meristems and shoot tips (Dore Swamy, et al., 1983; Cronauer and Krikorian, 1984; Vuylsteke and De Langhe, 1985; Wong, 1986). Production of callus cultures (Mohan Ram and Steward, 1964) and embryo-like structures have also been reported (Cronauer and Krikorian, 1983; Jarret, et al., 1985).

In this paper, we report the production of somatic embryos and subsequent recovery of plantlets from cultured zygotic embryos of the ornamental diploid banana Musa ornata (Roxb.) (Cheesman, 1949; Moore, 1957). These embryos are virtually indistinguishable phenotypically from zygotic embryos in that prior to germination, they form bulbous out-

growths reminiscent of the haustorium of a zygotic embryo.

MATERIALS AND METHODS

Immature fruits, approx. 4 cm long and approx. 11 weeks post-pollination, were harvested from greenhouse-grown banana plants (*M. ornata* Roxb.) The fruits were surface sterilized in 100 ml of sterile distilled water with 10 ml of commercial bleach (final concentration 0.47% NaOCl) and 3 drops of Tween 20 for 5 min and rinsed four times with sterile distilled water. Working under sterile conditions, the fruits were cut open and the immature seeds were removed. These seeds were cut open with a sharp scalpel and the embryos were excised and placed on semi-solid medium composed of the basal salts and vitamins of Murashige and Skoog (1962) (MS), 3% sucrose, 5 % coconut water (CW) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5, 1.2 mg/l, 2.25, 4.5, and 9 μ M respectively). The media were adjusted to pH 5.8 with KOH prior to autoclaving, solidified with 0.7% Difco agar and poured into 6 cm diameter sterile plastic petri dishes (Falcon #1007). The plates were sealed with Parafilm and maintained in growth chambers at 30^o C in darkness. Established embryogenic cultures were transferred to fresh semi-solid medium biweekly.

Germination was induced by transferring embryos to semi-solid medium composed of the mineral salts and vitamins of Schenk and Hildebrandt (1972) (SH), supplemented with 3% sucrose and 5% CW and placing them in a 18:6 L:D cycle. One month later, they were transferred to basal SH medium. Six weeks later they were transferred to basal MS medium. Continued root growth and the establishment of plantlets was obtained by placing embryos on filter paper bridges in liquid half-strength SH medium supplemented with 1% sucrose.

Histological sections were prepared by fixing material in Craif III, dehydrating it in a graded ethanol:Histoclear (National Diagnostics, Somerville, NJ) series, embedding in Paraplast (Monoject Scientific, St. Louis, MO) and sectioning at 8 μ m with a rotary microtome. Prepared slides were stained with aqueous saffarnin and Fast Green (Berlyn and Miksche, 1976). Material was

prepared for scanning electron microscopy by fixing in 3% glutaraldehyde in 0.03 M PIPES buffer, pH 8.0 at room temperature for 12 to 24 hr. It was then stored at 4° C until ready for viewing. Fixed specimens were dehydrated in a graded ethanol series on ice, critical point dried, mounted on stubs, sputtered with gold and viewed on an Amray 1000A scanning electron microscope at 5 Kv.

RESULTS AND DISCUSSION

Excised immature seeds were well formed and closely resembled mature seeds except that their seed coats were leathery and could be cut with a sharp scalpel. The endosperm was soft and gelatinous in consistency. The zygotic embryos, which were well formed and nearly as large as those found in mature seeds, were plated on the agar surface without regard to orientation. After approximately 20 days in culture, friable yellow callus began to form at the shoot apical meristem region of the zygotic embryo while the haustorium turned black (Fig. 1). As this callus grew, tiny somatic embryos began to emerge across its surface (Fig. 2). Histological sections showed that the callus was composed of isodiametric, cytoplasmically dense cells and lacked any organization or epidermal layers (Fig. 3). Somatic embryos formed in profusion when the tissue was subcultured at regular intervals (Fig. 4). All embryos emerged from callus tissue; no secondary somatic embryo formation was observed. Scanning electron micrographs showed that these somatic embryos were highly uniform in shape and possessed constricted bases and prominent notches (Fig. 5). Histological sections showed the apical meristem was located beneath this tissue (Fig. 6).

Germination of zygotic embryos of seeded Musa clones begins with the emergence of the primary root but it is soon overtaken by large numbers of adventitious roots. Subsequently, the plumule emerges (Fig. 7) and as the shoot develops, the first two leaves are essentially bladeless sheaths and the first expanded leaf blade forms on the third leaf (Simmonds, 1959; McGahan, 1961). When somatic embryos were transferred to basal SH medium, the tissue gradually became brown and the embryos increased in size. The prominent notch area became swollen and surrounded the

plumule like a collar. The distal end of the embryo became much more rounded and prominent and took on the appearance of the haustorium of a zygotic embryo (Fig. 8). Serial transverse sections showed that this tissue was composed of parenchyma cells and was delineated by a one cell thick epidermal layer. Vascular elements connecting it to the embryonal axis were clearly evident (Fig. 9). Subsequent sections revealed the apical meristem with 3 embryonic leaves (Fig. 10).

When somatic embryos were placed on MS medium, the first green leaves emerged through the notch. As in zygotic embryos, the elongation of the primary root was rapidly followed by the formation of many adventitious roots. However, very few germinated embryos continued to grow on basal MS medium. Subsequent root growth and the establishment of plantlets was achieved by placing germinated embryos on half-strength SH salts with 1% sucrose on filter paper bridges. The striking similarity of these plantlets to those derived from zygotic embryos was evident even at this stage since the first 2 leaves formed bladeless sheaths and the third leaf opened into the first leaf blade as described by Simmonds (1959, p. 260) (Fig. 11).

As has previously been described for a number of monocotyledonous species, especially in the Gramineae (Vasil and Vasil, 1986), somatic embryos formed in profusion in the presence of relatively high levels of 2,4-D. Although these embryos were much narrower than zygotic embryos (McGahan, 1961), the population was highly uniform in shape and cultures have continually produced large numbers of embryos since June 1986. The features in the anatomy and germination of somatic embryos that mimicked zygotic embryos as described by McGahan (1961) include: 1) formation of a bulbous haustorium-like outgrowth, 2) formation of a cotyledonary sheath surrounding the first leaves (Fig. 8), 3) the pattern of vascularization of the haustorium (Figs. 9 and 10), 4) the formation of a primary root (Fig. 8) followed by the rapid formation of many adventitious roots (Fig. 9), and 5) the absence of a leaf blade on the first two leaves.

The main Musa clones of commerce (the sweet dessert bananas which are generally

eaten raw and the more starchy plantains which are eaten cooked) are triploid and seed sterile. The production of callus from somatic tissue of triploid Musa clones with varying capacity to produce roots and embryonal forms has been described (Srinivasa Rao, et al., 1982; Cronauer and Krikorian, 1983; Cronauer, 1986). The formation in profusion of somatic embryos from zygotic embryo-derived callus establishes the use of this tissue as another starting point for regeneration. But the essentially seedless state eliminates this route as a strategy for production of somatic embryos from most economically important banana clones. Nevertheless, the existence and use of elite diploids and tetraploids in breeding schemes (Rowe, 1984) suggests that the procedures described here could be utilized in a similar setting.

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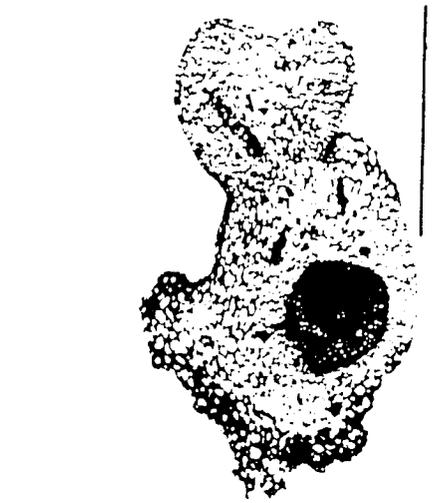
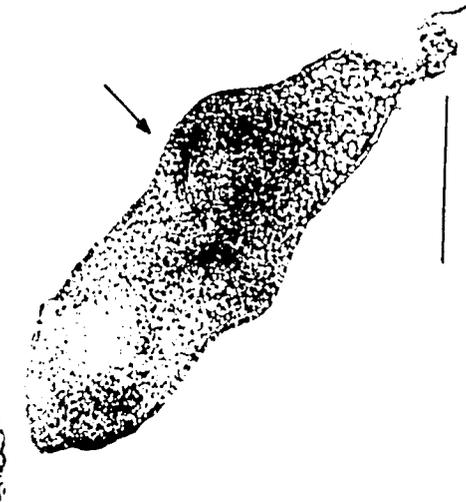
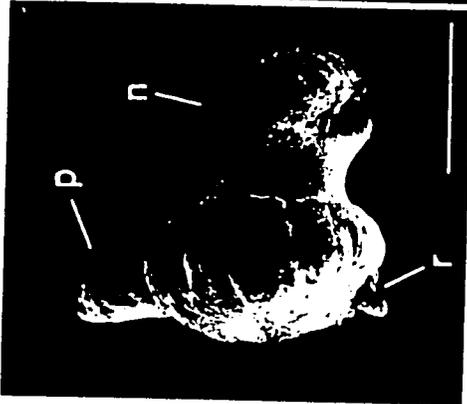
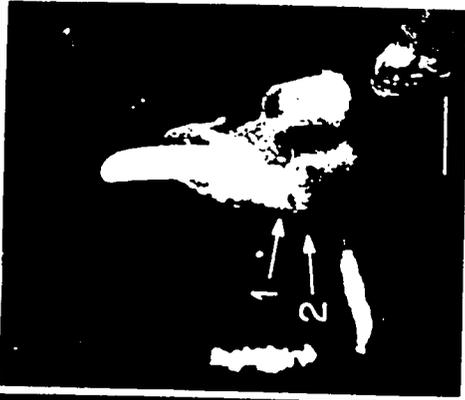
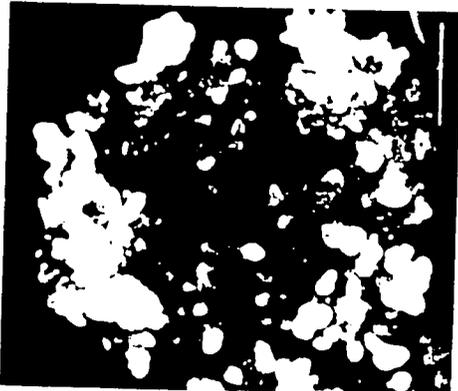
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Figs. 1-11. Stages in the initiation and germination of somatic embryos and subsequent plantlet formation in *M. ornata*. Fig. 1. Callus formation on a zygotic embryo cultured for 20 days on basal MS + 2 mg/l 2,4-D. Scale bar = 2.5 mm. Fig. 2. Early stage of somatic embryo formation (arrows) on proliferating callus cultures. Scale bar = 2.5 mm. Fig. 3. Longitudinal section of similar callus tissue showing compact cells and no epidermis. Scale bar = 0.1 mm. Fig. 4. Numerous somatic embryos. Scale bar = 2.5 mm. Fig. 5. Scanning electron micrograph of a single somatic embryo. Note the prominent notch (arrow) and constricted base. Scale bar = 0.25 mm. Fig. 6. Longitudinal section of a similar somatic embryo. The arrow denotes the apical meristem. Scale bar = 0.25 mm. Fig. 7. Scanning electron micrograph of a germinating zygotic embryo of a diploid banana *M. balbisiana*. Note the emerging plumule (p), radicle (r) and the bulbous haustorium (h). Scale bar = 1 mm. Fig. 8. Germinating somatic embryo of the diploid clone *M. ornata*. Note the young shoot, root and prominent haustorium-like outgrowth. Arrows 1 and 2 indicate the approximate positions of the serial transverse sections shown in Figs. 9 and 10. Scale bar = 1 mm. Fig. 9. Transverse section through a somatic embryo similar to that in Fig. 8 at arrow 1. Note the adventitious roots and vascular elements (arrow) connecting the "haustorium" to the embryonal axis. Scale bar = 1 mm. Fig. 10. Transverse section at arrow 2 in Fig. 8 showing a shoot apex and 3 young leaves. Scale bar = 1 mm. Fig. 11. Young plantlets rooted on 1/2 SH + 1% sucrose for 4 weeks. Scale bar = 20 mm.



Adventitious Shoot Production from Calloid Cultures of Banana

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ABSTRACT

Isolated tips (approx. 2 mm long) from aseptic, multiplying shoot cultures of the triploid dessert banana clone 'Highgate' were tested for their morphogenetic responsiveness to hormone treatments on semi-solid media. Medium containing Murashige and Skoog (1962) salts, p-chlorophenoxyacetic acid and kinetin produced a compact calloid mass. Protuberances disclosed by SEM as rounded, button-shaped and pointed outgrowths resembling fasciated shoots were formed in profusion. Sections showed many meristematic regions, some associated with distinct leaf primordia. Formation and growth of successive leaves yielded small, elongated adventitious shoots with constricted bases. Transferral to a basal MS medium with 1 mg/l NAA led to the formation of rooted plantlets.

INTRODUCTION

Historically, monocotyledons have generally been considered less responsive to callus induction techniques than dicotyledons. While there has been considerable success when efforts have focussed on the production of precociously branching or proliferating axillary shoot systems (cf. Hussey, 1980), it has generally been much more difficult to induce cell divisions with concomitant formation of a sustainable callus in many monocotyledonous species (cf. Hunault, 1979). However, work with various cereal species and some perennial monocotyledons like daylily, Hemerocallis, has demonstrated that progress in this area is now being made (Vasil and Vasil, 1986; Krikorian and Kann, 1986). Occasionally, a species will yield in culture a mode of growth that can be described neither as a true callus nor an organ culture and the term "calloid" has been used to describe it (Curtis and Nichol, 1948). In this paper, we report the production of a compact calloid mass from aseptically cultured vegetative apices of the dessert banana clone 'Highgate'. Although this tissue could not be described as an undifferentiated cal-

lus since it possessed an epidermal layer and mature vascular elements, it displayed numerous meristematic regions which subsequently yielded adventitious shoots and plantlets.

MATERIALS AND METHODS

Cultures-Shoot tips (approximately 2 mm long and comprising 2-3 leaf primordia) of three dessert banana clones, 'Philippine Lacatan', 'Grande Naine', and 'Highgate' (all triploid AAA according to the convention of Simmonds and Shepard, 1956) were excised from multiplying shoot cultures (Cronauer and Krikorian, 1984, 1986) and placed on medium comprised of the mineral salts and vitamins of Murashige and Skoog (1962), the iron of Singh and Krikorian (1980), 2% sucrose, 100 mg/l inositol, and growth regulators. The media were adjusted to pH 5.8 with KOH and HCl prior to autoclaving and were solidified with 0.64% Difco agar. Para-chlorophenoxyacetic acid (PCPA) (0, 0.05, 0.5 mg/l) and kinetin (0, 0.05, 0.5 mg/l) were tested in a Latin square arrangement. The apices were cultured, two per jar, in 35 ml French square jars with Bakelite plastic screw-on caps containing 15 ml of semi-solid medium in a growth chamber maintained at 30° C in continuous darkness. Cultures were transferred to fresh semi-solid medium at 3 to 4 week intervals.

Microscopy-Tissue pieces were fixed in Craff III, dehydrated in a graded ethanol: Histo-Clear (National Diagnostics, Somerville, NJ) series, embedded in Paraplast (Monoject Scientific, St. Louis, MO), sectioned 8 um thick and stained with aqueous Safranin-Fast Green (Berlyn and Miksche, 1976). Material was prepared for scanning electron microscopy by fixing in 3% glutaraldehyde in 0.03 M PIPES buffer, pH 8.0 at room temperature for 12 to 24 hr. It was then stored at 4° C until ready for viewing. Fixed specimens were dehydrated in a graded ethanol series on ice and critical point dried using a Sorvall Critical Point Drying System. The specimens were mounted on stubs, sputtered with gold and viewed on an Amray 1000A scanning electron microscope at 5 Kv.

RESULTS AND DISCUSSION

Apices cultured on medium containing 0.5 mg/l (2.68 uM) PCPA produced round, compact callus masses very similar to those described

by Cronauer and Krikorian (1983). In that work, similar tissue masses, lacking an epidermis, formed in close association with the vascular tissue on multiplying shoot clusters grown in a liquid MS medium containing 2,4-D or 2,4,5-T. Although the subsequent removal of auxin from the culture medium led to the production of roots, no shoots or plantlets could be recovered (Cronauer and Krikorian, 1983). Similarly, the transferral of compact callus to a medium lacking PCPA led to the formation of root structures which eventually blackened and died. When a lower level of PCPA was used (0.05 mg/l, 0.26 μ M), etiolated shoots with roots formed. However, in one case ('Highgate', 0.05 mg/l [0.26 μ M] PCPA + 0.5 mg/l [2.32 μ M] kinetin) a compact, pearly white calloid mass formed after 14 weeks in culture. This tissue was firm, not friable, did not blacken or otherwise discolor when subdivided with a few scalpel incisions, and was maintainable by subculture at 3 week intervals (Fig. 1). Cross sections revealed that this slow-growing tissue was composed of a vascularized parenchymatous ground tissue which was bounded by a distinct epidermal layer. Numerous meristematic regions were found under this epidermis (Fig. 2). These regions were composed of cells that clearly had undergone much division as demonstrated by the abundance and range of cells in various stages of mitosis. Longitudinal sections showed the emergence of distinct leaf primordia (Fig. 2). As the leaves enlarged and grew, these adventitious shoots became easily discernable to the naked eye (Fig. 3). The formation and growth of successive leaves led to the production of short, narrow shoots with constricted bases (Fig. 4). These shoots, which superficially resembled bipolar embryonic structures, protruded from the ground tissue and were easily separated from it by gently applying pressure with the dull side of a scalpel blade. However, sections revealed the presence of a clear vascular connection to the ground tissue and an absence of root primordia and hence confirmed their adventitious origin (Fig. 5). As these tiny shoots increased in size, adventitious roots developed at the shoot bases. Transferral of this material to a MS medium lacking growth regulators or a MS medium supplemented with 1 mg/l (5.37 μ M) NAA led to the formation of rooted plantlets (Fig. 6).

This calloid tissue was also noteworthy

for the large number of round protuberances visible across its surface (Fig. 7). Scanning electron microscopy showed that these outgrowths included pointed, stalked growth forms (Fig. 8) as well as slightly flattened protuberances with deep central depressions (Fig 10). Vascular connections to the ground tissue were clearly present (Figs. 9 & 11) but no prominent meristematic regions were seen although in one case, an area of small, dense cells was visible at the nadir of the depression (Fig. 11).

These unusual protruberances bear a striking resemblance to ring fasciations of axillary branches in tomato (Lycopersicon lycopersicum) (Gorter, 1965). Such fasciations can be induced by the external application of growth regulators to intact plant organs in situ. Nyman, et al., (1983b) also reported the production of fasciated leaf forms in taro (Colocasia esculenta var. anti-quorum) calloid cultures and we believe the stalked and rounded protuberances in our banana cultures represent a similar situation.

The production in vitro of differentiated cultures lacking distinct organs and defined as 'calloids' has been described in a number of monocotyledonous cultures including orchid (Cymbidium) (Curtis and Nichol, 1948) and taro (Nyman, et al., 1983a). In the case of taro, isolated apices grown on medium containing the auxin 2,4,5-T yielded callus which subsequently underwent a "transition" to a "stable" calloid growth form. This calloid tissue consisted of a vascularized ground tissue with many meristematic regions but lacked an epidermis. Our report here of a proliferating growth mode which is neither an epidermis-free callus nor an epidermis-bearing organ culture--specifically, a multiplying shoot tip--emphasizes the broad range of morphogenetic capabilities of a system such as Musa in vitro. The long term maintenance of such open-ended cultures in this calloid state which are capable of yielding many plants further emphasizes that banana plantlets can be regenerated from systems other than the branching axillary and adventitious shoot systems previously reported (Cronauer and Krikorian, 1984, 1986; Vuylsteke and De Lange, 1985; Wong, 1986). It also re-emphasizes the importance of histological sections to substantiate the origin of regenerated materials.

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Figs. 1-6. Adventitious shoot production from calloid tissue of the banana clone 'Highgate'. Fig. 1. Calloid derived from a shoot tip grown on MS + 0.05 mg/l PCPA + 0.5 mg/l kinetin for eight months. Scale bar = 5 mm. Fig. 2. Cross section of a piece of 'Highgate' tissue showing several adventitious meristematic regions and one well formed shoot meristem. Scale bar = 1 mm. Fig. 3. Scanning electron micrograph of an emerging shoot apex. Scale bar = 0.1 mm. Fig. 4. A cluster of short, narrow adventitious shoots with constricted bases. Scale bar = 5 mm. Fig. 5. Longitudinal section of a shoot as in Fig. 4. The adventitious nature of the young shoot is confirmed by the vascular connection to the basal tissue. Scale bar = 1 mm. Fig. 6. Adventitious shoots allowed to root on MS with no hormonal additives. Scale bar = 5 mm.

Figs. 7-11. Fasciated shoots on 'Highgate' calloid tissue. Fig. 7. Scanning electron micrograph of calloid tissue grown on semi-solid medium. Note the numerous rounded and pointed protuberances visible across its surface. Scale bar = 1 mm. Fig. 8. Micrograph of a pointed, stalked growth form. Scale bar = 0.1 mm. Fig. 9. Longitudinal section of a similar growth form showing the central vascular tissue in the stalk. Scale bar = 0.5 mm. Fig. 10. Micrograph of a flattened growth form with a deep central depression. Scale bar = 0.2 mm. Fig. 11. Longitudinal section of a similar growth form. Scale bar = 0.5 mm.





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DETERMINATE FLORAL BUDS OF PLANTAIN (Musa AAB)
AS A SITE OF ADVENTITIOUS SHOOT FORMATION

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ABSTRACT

Floral buds of the 'False Horn' plantain clones Musa (AAB) 'Harton', 'Harton Verde', 'Harton Negra', and 'Curare' terminate in a large single floral structure. The apices of these floral buds are here designated as determinate since they have lost the ability to produce additional floral initials or buds. Terminal peduncle segments can be cultured in a modified Murashige and Skoog (1962) medium supplemented with N⁶-benzyl-aminopurine (5 mg L). Under these conditions, this apparent inability to yield buds can be overcome as vegetative shoot clusters form in the axils of the bracts. Rooted plantlets are obtainable by treating shoots with naphthaleneacetic acid (1 mg/L) and activated charcoal (0.025%). The adventitious origin of the shoots has been established.

Key words: Musa cultivars, plantains, floral bud, adventitious buds, tissue culture.

INTRODUCTION

Plants of the genus Musa, the bananas and plantains, are large herbaceous monocotyledons which grow from massive underground corms. The transition from vegetative to floral growth and subsequent fruit formation marks the end of growth for an individual shoot meristem. For all practical purposes, a given shoot behaves like a monocarpic plant. The first floral organs to form are the bract-covered hands of female flowers the ovaries of which will become the fruits. In the dessert bananas (i.e. those clones with sweet fruits which are frequently eaten raw) and the so-called 'French plantains', the floral apex can continue to produce large numbers of bract-covered male flower clusters until the fruit bunch is harvested (Barker and Steward, 1962; Simmonds, 1966). In those clones designated as 'False Horn' plantains (with long horn shaped fruits generally eaten after cooking), the axis of the male inflorescence terminates in a large single flower and usually withers away soon after all of the fruits have formed (De Langhe, 1961; Tezenas du Montcel, De Langhe and Swennen, 1983). Because the dessert and French plantain clones have, in theory at least, the morphological capacity for continued formation of primordia on the flanks of their floral apical meristems, it seems appropriate to refer to such apices as indeterminate. Similarly it is appropriate to refer to those floral bud types where it seems an apparent morphologi-

cal impossibility to form additional bract or leaf primordia and buds on the terminal structure as determinate.

Using the dessert banana clone 'Dwarf Cavendish' as a prototype, we described the means whereby isolated terminal indeterminate floral apices could be induced to re-initiate a vegetative growth mode directly without the formation of an intervening callus (Cronauer and Krikorian, 1985). In this paper, we report that when the distal segment of the determinate inflorescence of a 'False Horn' plantain is cultured in a cytokinin-containing medium, adventitious vegetative shoots can be induced to form in the axils of the bract scars. Once this occurs, the system behaves and can be manipulated like a vegetative apex-derived culture.

MATERIALS AND METHODS

Terminal flower buds of three 'False Horn' type plantain clones, 'Harton', 'Harton Verde', and 'Harton Negra' of the 'Harton' type [AAB genome group according to the convention of Simmonds and Shepherd, (1956)] were collected in Maracay and Santa Barbara del Zulia, Venezuela as were corms of 'Harton Verde' and 'Harton Negra' (cf. Haddad and Borges, 1973). Flower buds of the common 'False Horn' plantain known in Costa Rica as 'Curare' were collected in La Lola, Costa Rica. In the case of the Venezuelan material, the flower buds were

carried from the field to a laboratory in Caracas. The majority of the bracts were removed by hand until the buds measured approximately 6 to 7 cm in length. They were then surface sterilized in a 2% (v/v) commercial bleach (Clorox) solution (final concentration 0.1% NaOCl) for 5 min and rinsed four times with sterile distilled water. They were placed on 10 ml of semi-solid medium in 18 mm X 150 mm Kaput tubes (Bellco Glass, Inc., Vineland, N.J.) which were sealed with Parafilm and hand carried to Stony Brook. The culture medium was composed of the mineral salts of Murashige and Skoog (1962) and iron of Singh and Krikorian (1980). 100 mg/L inositol, 1 mg/L thiamine HCl, 5 mg/L N⁶-benzylaminopurine, 4% sucrose, and 0.7% agar. The pH of the medium was adjusted to 5.8 with KOH prior to autoclaving at 121 C and 103 kPa for 20 min. Upon arrival in Stony Brook, the entire flower buds were placed in 50 ml of liquid culture medium of the same composition in 250 ml Erlenmeyer flasks and placed on a gyrotary shaker at 100 rpm in a growth chamber maintained at 30 C under a 16:8 L:D cycle (10.2 x 10³ lm/m² Sylvania Grolux wide spectrum). The corms were hand carried to Stony Brook where vegetative apices were isolated as previously described (Cronauer and Krikorian, 1984, 1986). The Costa Rican material was hand carried in an insulated cooler from the field to Stony Brook where they were prepared as described and placed directly into liquid medium. Multiplying shoot

clusters were removed from the floral axis and transferred to semi-solid medium in 6.5-cm x 6.5-cm x 10-cm plastic vessels (GA 7 vessels from Magenta Corp., Chicago, IL) and were routinely subcultured at 3-4 wk intervals. Roots were induced to form on individual shoots by placing them on 10 ml of semi-solid medium containing 1 mg L naphthaleneacetic acid and 0.025% activated charcoal in Kaput tubes.

Material isolated for histological examination was fixed in Craif III (Berlyn and Miksche, 1976), embedded in Paraplast, sectioned at 8 um, and stained with an aqueous safranin-fast green series. Material was prepared for scanning electron microscopy by fixing in 3% glutaraldehyde in 0.03M PIPES buffer, pH 8.0, at room temperature for 12 to 24 hr. Fixed specimens were dehydrated in a graded ethanol series on ice, critical point dried, mounted on stubs, sputtered with gold and viewed on an Amray 1000A scanning electron microscope at 5 Kv.

RESULTS AND DISCUSSION

Flower buds of determinate Musa clones are composed of many layers of tightly clasping purple bracts and are much more elongated than those of indeterminate clones (Fig. 1). When the outermost bracts were removed, occasionally a "hand" composed of 2 or 3 fruits was encountered but most of the bracts lacked the expected

associated nodal cluster of fruits. No nascent flower promordia were visible to the naked eye (Fig. 2) or in sections. The removal of the most distal bracts revealed a large terminal flower structure composed of four stamens and a pistil (Figs. 2 and 3).

During the first six weeks in culture, flower buds were transferred to fresh liquid medium every 7 to 10 days due to the rapid blackening [presumably phenolic in origin (Palmer, 1963)] of the medium. The remaining bracts turned brown during this period and were eventually removed, thus revealing the pistil and stamens which eventually blackened and either atrophied (Fig. 4) or were surgically removed. The distal portion of the peduncle remained green while the cut surfaces which resulted from the removal of all bracts blackened.

Shoot formation began with the appearance of bud-like structures in the axil of the bract scars (Fig. 5). As these buds grew in size, they became green and, in some cases, the outermost leaf took on the appearance of a miniature bract in that it did not completely encircle the apical growing point the manner of a vegetative leaf. However, with subsequent leaf formation, shoot morphology became indistinguishable from that of shoots originating from cultured vegetative apices. This response usually occurred at most of the nodes along the floral axis (Fig. 4). As the tight clusters of shoot buds expanded, they encircled the

floral axis and later were separated from it with a few scalpel incisions.

Overall shoot morphology at the multiplication stage appeared to be identical to that of material originating from vegetative apices (Fig. 6). Root formation proceeded in a similar manner for all shoots regardless of their origin (vegetative vs. floral) with the first roots appearing 5 days after shoots were placed on auxin-containing medium. Within three weeks, the young plantlets were large enough to be transferred to the greenhouse where plantlets of vegetative and floral origin have been established.

In triploid (AAA) dessert banana and French plantain (ABB) clones with floral buds of the type here designated as indeterminate, explanted flower buds (order of 2 cm in length) possessing numerous hand initials or primordia can be induced to grow and eventually give rise to adventitious shoot buds. The resulting cultures usually contain many diverse floral structures ranging from ovaries to entire floral buds several cm in length. Multiplying shoot cultures can be obtained by repeated selection and subculture of the desired material (Bakry, et al., 1985; Cronauer, 1986). In the case of cultures initiated from primordia-free apical domes, a vegetative growth mode can be induced directly without the formation of callus or floral structures (Cronauer and Krikorian, 1985).

The relative ease with which plantlets could be recovered from indeterminate flower buds prompted us to examine the responsiveness of determinate flower buds of false horn plantain clones since the tissues of these structures are fully committed, i.e. there are no floral or vegetative primordia present. In all four clones tested, it was possible to induce vegetative bud formation and growth without the concomitant production of floral structures even though these vegetative buds formed at the putative site of a floral organ.

The site of origin of these shoot cultures implied an adventitious origin and this was confirmed by histological examination of peduncle segments from noncultured flower buds as floral primordia were absent from the axils. Examination of cultured peduncle tissue showed adventitious bud formation (Fig. 7) and subsequent growth (Fig. 8). The production of shoot buds at this anomalous location indicated that the cells in this area were responsive to treatment with an appropriate growth regulator, in this case, a cytokinin. Previous reports on the culture of flower stalk segments in monocotyledonous species have dealt with the recovery of plantlets from pre-existing buds in the orchids Dendrobium and Phalaenopsis (Arditti, 1977), as well as the de novo formation of vegetative buds in Narcissus (Seabrook, Cumming, and Dionne, 1976) and Hosta (Zilis and Zwagerman, 1979), and bulblet for-

mation in Hyacinthus (Paek, 1982).

In commercial plantations, clonal multiplication of Musa species is sometimes induced by stripping away the outer leaf sheaths of the pseudostem and inducing the otherwise dormant leaf-opposed buds to emerge (Barker, 1959). Presumably, in vitro multiplication of shoot cultures proceeds in a similar manner with the use of high levels of cytokinin (order of 5 mg/L) inducing pre-initiated bud growth (Dore Swamy, et al., 1983; Cronauer and Krikorian, 1984; Vuylsteke and De Langhe, 1985; Wong, 1986). However, adventitious shoot formation can also occur. Histological sections (Fig. 8) and scanning electron micrographs (Fig. 9) have revealed that the site of the vegetative meristem of a single cultured shoot can contain multiple apices in close association with each other (Banerjee, Vuylsteke, and De Langhe, 1985; Cronauer, 1986; Cronauer and Krikorian, 1987). The implications of adventitious shoot formation on the performance and clonal fidelity-stability of this cultured plant material in the field has yet to be assessed.

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Fig. 1-6. Some representative stages in the initiation and culture of vegetative shoots and subsequent plantlet production from floral buds of plantains of determinate floral type. 1. Terminal floral bud removed from the distal end of a 'Harton' fruit bunch. x 0.38. 2. The same bud with bracts removed to reveal the peduncle and terminal floral structure. x 0.48. 3. Close-up of a 'Harton Verde' floral structure. The four stamens surround the shorter, bent pistil. x 1.8. 4. Formation of vegetative 'Harton Negra' shoot buds in the axils of the bract scars along the peduncle. Note the withered terminal flower structures (arrow). x 1.7. 5. Vegetative bud formation at the distal end of a 'Curare' peduncle segment. The removal of the terminal floral structures has left a large, prominent scar (sc). br=bract scar. b=vegetative bud. sh=shoot. x 3.6. 6. Shoot cluster derived from cultured 'Harton Verde' floral bud. x 1.3.

Fig. 7-10. Adventitious shoot formation in cultured vegetative and floral tissues of plantains. 7. Longitudinal section showing the formation of an adventitious 'Curare' bud in the axil of a bract. x 90. 8. Later stage in adventitious shoot growth in the clone 'Curare'. x 75. 9. Slightly oblique longitudinal section of a 'Cardaba' (ABB) shoot derived from a cultured vegetative shoot tip which shows the formation of multiple shoot primordia. x 56. 10. Scanning electron micrograph of 'Cardaba' material similar to that in Fig. 9. Note that the outermost leaf primordium surrounds both shoot primordia. x 87.

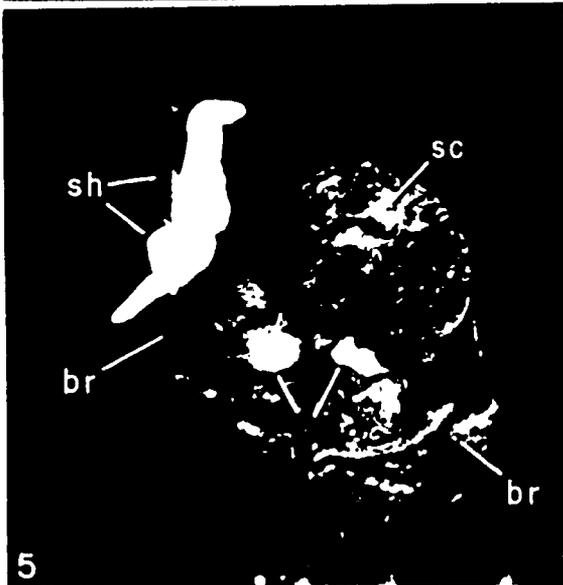
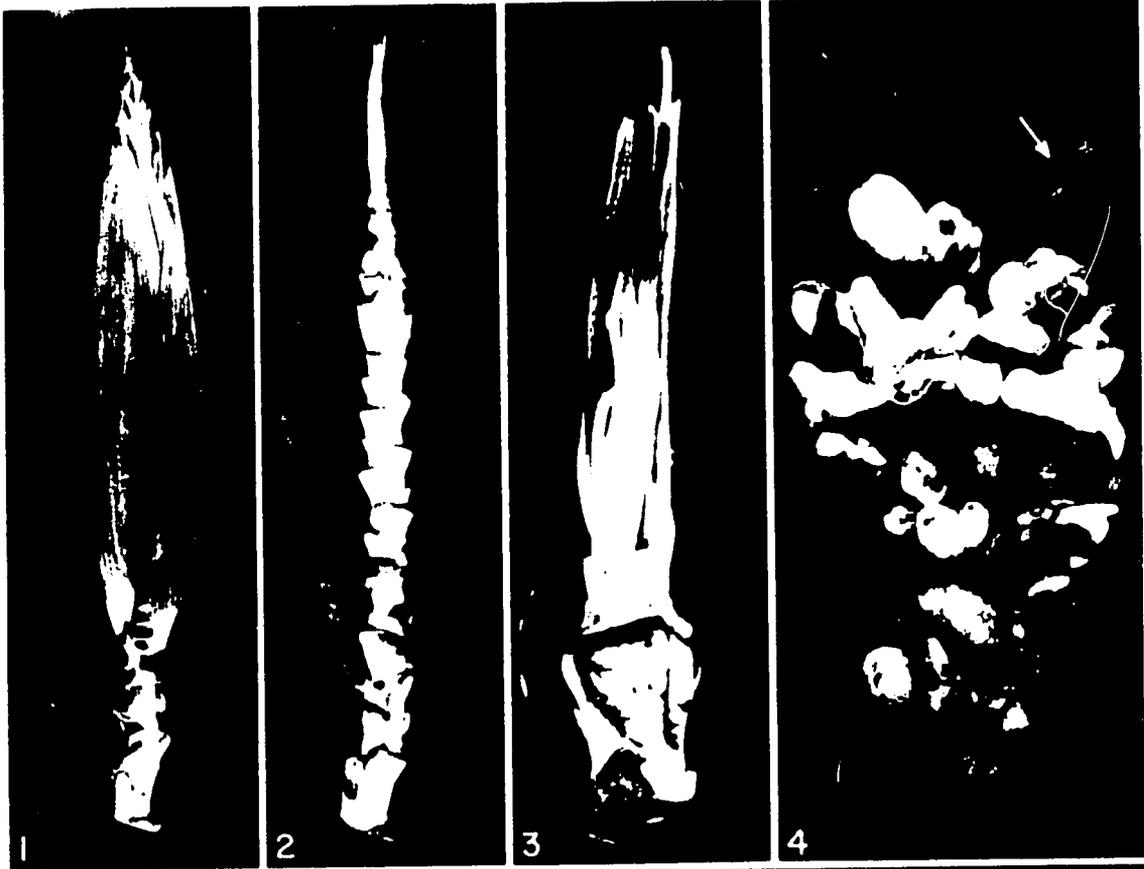
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Musa TISSUE CULTURE: Dilemma or Challenge?¹

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Introduction

Much has been presented at meetings and written about tissue culture of bananas and plantains and the role it might play in improvement of this important crop. The perception of what has been achieved is at variance, in my opinion, with the reality. Some claims are more easily seen as sins of enthusiasm rather than reflections of scientific consensus but the end result is the same. The fact is that we are only at the beginning. Unfortunately, in spite of some totally predictable, and indeed anticipated (by some investigators at least) problems, we are now in the dangerous predicament of potentially experiencing an eroding base of support for banana tissue culture efforts.

There is a perception on the part of some (viz. those who are called "experts" but are usually quite inexperienced in the field) that the techniques are essentially in place and should now be used to produce a product. Yet another erroneous perception, at the other extreme, is that tissue culture procedures for Musa, because of the special problems posed by the plant, cannot yield anything really significant in a reasonable period of time. This is based in part on the projections of some that

Footnote 1. This is an expanded version of a manuscript in Spanish (Krikorian, 1987d).

the basic precepts for selection of leaf spot disease-tolerant clones are ill-conceived (cf. e.g. Buddenhagen, 1987; Daub, 1986).

As usual, those views which espouse either extreme are erroneous and the truth lies somewhere in between. This article seeks to state, once and for all, where I feel we are and where we should be going.

The opportunities offered by judicious use of aseptic culture technology for plant improvement have been stated and re-stated many times. Conservative workers have always appreciated the difficulties associated with controlled use of any tissue culture procedure, much less those techniques related to modern biotechnology and so-called genetic engineering. A major constraint to the effective formulation of research and development thrusts and initiatives in all places has been, in part, failure to appreciate fully the limitations as well as the capabilities of the available methodologies. In defense of all concerned, it should be emphasized that the "new" biotechnologies are naively being heralded virtually everywhere as offering an ultimate guarantee of realizing broad, sweeping solutions to the world's agricultural production and crop "improvement" problems. Like other evolving methodologies and trends, it is often difficult to separate possibility from achievement, and expectation from fantasy. There is no doubt that aseptic culture techniques offer powerful tools for investigation, and in some few cases, for development, but we all have to learn much more about basic biological facts as they relate to any given "system" before we can even begin to expect routine implementation at the laboratory level. The challenge,

therefore, in Central and South America, and the Caribbean--indeed everywhere--is and will continue to be for quite a few years, to identify realistic and significant projects with a reasonable level of promise and potential for pay-off in a practical setting. This article begins by attempting to show how scientific involvement often originates, how a scientific problem evolves and develops even as progress is made, only to disclose new problems and demand even more innovative and coordinated approaches. Although I use tissue culture of Musa as the focal point of this paper for UPEB's Informe Mensual, the same problems would exist to varying degrees if other plants were to be used for crop improvement schemes.

Bananas and Plantains and Tissue Culture

My laboratory at Stony Brook embarked upon a broadly based tissue culture of Musa effort because of our interest in the problems of growth and development, and our expertise in aseptic culture methodologies. A long-standing interest in monocotyledons, especially perennial ones, and early exposure to tissue and cell culture of banana initially that of fruit, at Cornell University while I was a pre-doctoral student in F. C. Steward's laboratory from 1959 to 1965, and much later, through close friendship with the now late Emerita de Guzman (1929-1981) of the University of the Philippines at Los Banos (UPLB), with shoot tip methods--provided additional bases of incentive.

For reasons that I need not go into here, my original hope was to have collaborated on a Musa cell culture project with Dr. de Guzman and her associates, especially Aurora G. del Rosario. When Emerita died of cancer, it seemed all the more important to carry on the work--especially from the perspective of those aspects of the problem which were sure to be more

demanding of facilities not then or widely available in most developing countries. This meant first refining and extending the shoot tip methods, and then attempting callus, cell, and protoplast culture as preludes to more biochemical and genetic approaches. "Bing" del Rosario, has over the years, been working on and extending the coconut tissue culture work initiated by Dr. de Guzman and herself and has also shouldered the main responsibility of the tissue culture work with abaca (manila hemp), Musa textilis. Shoot tip culture work on edible Musa clones was shifted after Dr. de Guzman's death from the Department of Horticulture, UPLB, to the Institute for Plant Breeding, UPLB, where there is some work on several fronts including germ plasm preservation and maintenance for eventual exchange etc (Zamora et al., 1985). Initiative for a banana-plantain mutation breeding project has also happily been revived and is now centered in Vienna at the International Atomic Energy Agency under the direction of Dr. F. Novak (cf. Menendez, 1973; Novak et al., 1986).

I knew at the outset of our studies that aseptic culture procedures for banana, as I envisioned them, would not be an easy undertaking. Our successes with two perennial monocotyledonous systems, namely the daylily, Hemerocallis (cf. Krikorian et al., 1986) and oil palm, Elaeis (cf. Krikorian and Kann, 1986), however, served as an encouragement. Most of the available literature on Musa, limited as it was, unfortunately, was not detailed enough to be very helpful to us. (There are some hints in the literature that some callus culture was attempted in a couple of commercial plantation laboratories (e.g. United Fruit) but the work turns out to have been very preliminary.) The work of Mohan Ram and Steward, essentially "wound down" in 1961 but not terminated until 1963, was, therefore, all we had for leads into callus and cell culture work (Mohan Ram and Steward,

1964). Fortunately, I had the advantage of having had first-hand experience with those cultures from "student days." The approach exemplified by the published work of de Guzman, Ubalde and del Rosario (1976) provided good background and guide to the shoot tip culture work. Also, thanks to a U.S. National Science Foundation International Programs Division-funded trip to the Philippines aimed at fostering collaboration, I had been shown the shoot tip technique, and even returned to Stony Brook with materials in vitro. The work of Ma and Shi published in Chinese with brief English summaries (1972, 1974), was not then known to me. The work of Berg and Bustamante (1974) said to be stimulated by the Chinese work did not, so far as I could tell, involve multiplication. Their emphasis was to show that cucumber mosaic virus (CMV) could be eradicated by in vitro methods. Only one plant was obtained per excised shoot tip (Berg and Bustamante, 1974). All this emphasizes that more often than not, one learns for oneself how to manipulate a system, or one is taught the "tricks". It is certainly easier to have prior exposure and guidance on how to proceed. Much wastage of time and grief can be avoided. Many citations in the current tissue culture literature dealing with Musa very generously (and inaccurately, in my view) give or imply too much credit to those who published first on shoot tip culture. That work, like much of the work on bananas, was generally inaccessible, turned out to have used very large explants which can hardly be called "shoot apices", lacked critical detail and could therefore not be very helpful in my opinion to any would-be investigator seeking to carry out meristem work. In my view, the UPLB work was the work that first made the plant tissue culture community aware of what could be done with a Musa shoot tip system. Dr. de Guzman made several presentations over the years, in different countries,

on banana (and coconut) tissue culture work from the perspective of shoot tip multiplication and mutagenesis for plant improvement. Since her laboratory project on banana was funded in part by the International Atomic Energy Agency, the formal proceedings of the working sessions of that group were the prime forum for publishing her progress reports. The international make-up of those working groups indicates the large and geographically diverse professional plant science community who knew about the UPLB work. Also, there were plenty of visitors to Dr. de Guzman's laboratory in the Horticulture Department at UPLB. A poster of the banana work personally presented by Emy at the 4th International Congress of Plant Tissue and Cell Culture at Calgary, Canada in 1978 brought the work into further prominence.

It is important to give credit where credit is due, but it is another matter to suggest that chronologically "first" papers are necessarily critical to later thinking and achievements. I learned Musa shoot tip culture from the UPLB laboratory, refined it, extended it to meristem culture with Sandra Cronauer and my laboratory has taken that as the 'base line' for our newer work. On every occasion I have taken meticulous care to acknowledge sources of support and to give full credit where I thought it was due from our perspective. In the process of all our work, we have also been very active in making the essentials of the banana shoot tip and meristem technique available to all those interested (cf. e.g. Angarita Z. and Castro, R., 1984; Perea Dallos et al., 1985). (Indeed, sometimes the burden of time spent and cost of communication with people whom I do not even know has been exasperating.) "Pre-prints" of the article published finally in the Handbook of Plant Cell Culture (Krikorian and Cronauer, 1984) were made available by me as early as 1981.) Some of our

publications have gone to the extreme of providing step-by-step protocols (cf. Krikorian and Cronauer, 1984a; Cronauer and Krikorian, 1984b, 1985b,c 1986b). Sandra S. Cronauer, first as a graduate student, and more recently as a Post-doctoral Research Associate, has played a major role in the Stony Brook project. We have worked closely together and hard, with little funding. Mary Scott, a laboratory technician, has been a major contributor for she maintains, very effectively, the large germ plasm collection stocks of shoot tip cultures. At every stage of the project she has been a direct source of support since Musa tissue culture work is very labor-intensive. Over the past few years, Mrs. Scott has also been working very closely with me on developing more demanding technology - namely callus systems in liquid and cell suspensions (cf. Krikorian, 1987c). David L. Smith, a graduate student who is working on cell culture systems other than Musa, has also been involved since he has taken much of the initiative in developing histological and microscopic techniques for Musa. Mr. G. David Whitmore, a highly trained and experienced horticulturist and Curator of Plant Growth Facilities at Stony Brook, has been of considerable help for he has taken on much of the responsibility of maintaining our "glasshouse and growth chamber Musa germ plasm." Mr. Robert P. Kann, a long-standing 'Technical-Specialist' in my laboratory has also played a significant role since he, too, helps maintain the germ plasm ex vitro. Last but not least by any means is the role played by our Senior Laboratory worker Suzanne del Villar. Mrs. del Villar has the demanding responsibility, among others, of maintaining mountains of laboratory glassware etc. scrupulously clean. In short, it must be recognized that a very capable and broadly-based, minimum critical mass of personnel is necessary to attack the problems posed by Musa tissue culture. All too

often, aspiring investigators, and certainly those whose responsibility it is to award and "manage" grants, do not appreciate that even a modest project very quickly undergoes escalation and becomes a multi-faceted effort. None of our plantain-banana work would have been possible if there had not been in place from other sources of funding a major tissue culture facility complete with trained personnel and operating budget. A feature of the Stony Brook work is that experiments have been done over and over again so that our publications do not reflect sporadic or careless work. The work is reported in such a way that it is repeatable. This aspect of reliability is crucial if Musa culture work is to go forward. Things are not so easy or straightforward as some who have published what are to me "curious" results would have us believe (cf. e.g. Gupta, 1986).

While one can make decisions to limit oneself completely to one facet of a project, this is inevitably unwise -- especially when a new and difficult project is undertaken. One needs to "cast a wide net" in order to ascertain the potentials, capabilities and limitations of a system. This means carrying out careful probes "here and there", and then following through or pursuing those that are most promising. This means keeping an "open mind" and "going with the system". Barbara McClintock, Nobel Laureate, has given this approach a name. She calls it developing "a feeling for the organism".

One does not develop a feeling for the organism if the investigation is too narrowly conceived, or proscribed, or circumscribed. The other side of the coin is, however, that one can only do so much with the resources at one's disposal. In the end, it inevitably becomes a compromise.

If all the above is so, as I believe it to be, then the problem is one of deciding what kind of tissue culture project will be a scientifically

rewarding one. One must be sure, however, that one is interested in either science, or technology, or both. It helps to appreciate that technology cannot be applied without a scientific base from which one can depart.

Science can be done by anyone who is a scientist. There are many excellent science projects that can be undertaken by individuals with virtually no resources. To undertake the application of science for practical ends, requires a substantial group effort and financial support. To me this is a point much unappreciated and much misunderstood.

From my perspective, I believed at the outset of our studies and still believe, that there is considerable potential for safe-guarding our plantain-banana resources, and for banana, plantain and cooking banana improvement by use of meristem, tissue, cell, and protoplast culture techniques. The fact still is, however, that the methods must be developed. It will take a lot of time to do this. I said so and that view has not changed. Musa has always been a very challenging system to work with. At present, it is accurate to state that we do not have the techniques in hand to do routine practical work except with meristems and shoot tips! The challenge of working with such a difficult system is attractive to me. It is all well and good for some to state that the problems are technical, but anyone experienced in such matters knows that the problems are fundamental and basic. While some say tissue culture is an art, rather than a science, the fact is that tissue culture work when analyzed carefully can show, very elegantly, and at close hand, the very nature of the problems of understanding growth and development. To call such work "technology", does a major disservice to the problems one has to understand and surmount before one has a usable, working aseptic culture "system." In fact, one is only now beginning to reach this point in some

plants. Unfortunately, banana is not yet one of them. My sincere hope is that one day it will be.

Seedless, (edible) bananas and plantains are, of course, ordinarily parthenocarpic and effectively seed-sterile. Breeding techniques have for all practical purposes been unproductive in the past because of difficulties of working with essentially seedless plants. Compounding the difficulties of relatively limited and clonal or largely unchanged germ plasm is the fact that these plants are often grown as monocultures which persist for a long time. Diseases therefore have a critical impact. Since any breeding effort is seriously limited, and takes enormous amounts of time (Rowe, 1981, 1984; DeLanghe, 1987; Simmonds, 1987), I feel it is critical that we evaluate the potential of newer techniques such as plant tissue culture to shorten the length of time involved in generating new and improved Musa clones. It may turn out that the time needed to do anything with aseptic culture will even exceed that needed for conventional breeding but that does not mean we should not make it our goal to learn how to handle Musa in vitro.

In all of my talks and writings, I have aggressively adopted what I feel is a realistic, conservative, research approach to tissue culture techniques and Musa improvement (Krikorian, 1982, 1987a,b,c; Krikorian and Cronauer, 1982a, 1984b). Time and time again I have emphasized that we must go step by step. We must walk before we run. At best, the methods will be adjuncts and supplements to breeding and improvement procedures. That is to say, breeding, even in a recalcitrant crop like bananas and plantains, will more than likely not be superceded (cf. Simmonds, 1983)! On the other hand, Musa is an excellent candidate, probably one of the best I can cite, for tissue culture manipulations. Such work can open a whole

new vista on dealing with problems of improvement of not only Musa, but other vegetatively propagated plants as well.

Shoot tip and Vegetative and Floral Meristem Procedures for Musa

The methods for rapid multiplication are now partly worked out--at least in principle. The strategy includes: 1) collection of elite field grown material; 2) preparation of primary explant material; 3) surface disinfestation and decontamination and preferably, virus elimination; 4) excision of the explants as meristems or stem tips; 5) exposure to growth regulator treatments in vitro in an appropriate culture medium so as to stimulate shoot bud proliferation; 6) testing for asepsis; 7) further multiplication and separation of the shoots rigorously demonstrated as clean and further subdivision of them (in any of several ways) so as to stimulate more shoots; 8) separation and rooting of the individual shoots so as to regenerate plantlets; 9) removal of the plantlets from in vitro to ex vitro with the view of acclimating them for field planting; 10) and finally, field evaluation. Figure 1 provides a schematic diagram of our shoot tip and meristem, both vegetative and floral, procedures. The many steps involved emphasize the many places where variations and modifications in the methodology are possible. If carefully thought out, each of these presents research opportunities. In my laboratory, there is, and will continue to be, so long as some research funds are available, an ongoing effort to improve and evaluate the methodologies (cf. Krikorian and Cronauer, 1984a; Cronauer and Krikorian, 1984b, 1985b,c; 1986b; 1987).

At Stony Brook, I have maintained comprehensive files and records of correspondence with many investigators from all over the world who have done, or are carrying out tissue culture on Musa and related groups. The important feature that emerges from all this is that there are many

variations on the above theme (cf. Mascarenhas et al., 1983; Vuylsteke and de Langhe, 1985; Sandoval, 1986; Wong, 1986). There are quite a few ways to achieve a given end and what appears best for one laboratory, is not necessarily seen by others as best for them. The critical point, however, is that there is a very real potential for what I call "tissue culture management problems" to emerge and, indeed I predict that there is a direct relationship between the aseptic culture protocols and the way one carries them out and the end product. What I am saying is that one reason, among a possible range, for somaclonal or tissue culture-associated, or tissue culture-derived variation is the way the tissue culture is carried out. It is perhaps too idealistic to think that a consensus will ever be reached on the "best" in vitro methods to be followed.

Even so seemingly simple a matter as routinely carrying out tests to ensure that nominally aseptically-generated cultures are indeed aseptic has been ignored by nearly all investigators. Far too many laboratories assume that cultures are "clean" because they show no "obvious" contamination of the medium. It is easy enough for an inexperienced person to miss this especially if agar based medium is used. The fact is that one must cut and expose tissues to media suitable for both bacterial and fungal microorganisms (e.g. nutrient broth and agar, and Mycophil broth and agar), and to incubate at elevated temperatures (ca. 35°C). Simple plant nutrient culture media, and cultures grown at non-permissive temperatures are generally insufficient to disclose cryptic contaminants. Spore forming bacteria are among the worst offenders. Use of Gel Rite (gellan gum, Kelco, San Diego, CA) in place of agar can be of some use to disclose contaminants since it is more translucent, but we do not recommend its use for routine culture because of its adverse effects on other culture

characteristics. We routinely screen cultures for infection. Some investigators seem to be preoccupied with various medium components (especially growth regulators) in their "protocols" to the seeming exclusion of everything else. Speed and numbers are important but it is important to view the entire system holistically. For us, and in connection with the shoot tip and related systems, "management" has become very important since growing things in quantity in discrete stages allows one to study development and to make meaningful comparisons and draw conclusions. One can argue ad infinitum the pros or cons of this or that medium supplement, or culture vessel, or whether rooting should be carried out as a separate step, or not etc. etc. These will, in part, depend on what the perceived needs and priorities are. The "elegance" or "cleanliness" of the morphogenetic response associated with a given protocol is of great importance to us, but it is not the only thing at stake. This point will be elaborated upon later.

In the course of our studies on strict meristems (i.e. apical domes lacking primordia) we have also learned that excised floral meristems as well as vegetative ones can undergo a "re-programming" and yield vegetative shoots in large numbers (Cronauer and Krikorian, 1985a,b; 1986a,b; 1987). The technical advantage, which has great theoretical interest and which still remains to be proven as a real practical advantage, is that the apical dome of the floral axis of desert and other bananas, is easier to excise because it is elevated and thus, accessible to surgical manipulation. Vegetative shoot apices are much more difficult to excise as strict apical domes since they are located in a more or less "depression." The possibility of being able to work with the meristematic dome is a special advantage where one is concerned with questions relating to

developmental plant biology. There is also the potential advantage of possibly being able to obtain directly, a meristem free of specific pathogen such as virus, if one can excise a very small dome. (This is yet to be demonstrated, conclusively, however). We are only now doing field studies to compare floral meristem- and vegetative meristem-derived plants.

Over the last few years, a relatively large body of literature which relates to the aseptic culture of bananas and plantains has emerged. Cronauer and Krikorian (1985b) provide a table which analyzes all this literature from the perspective of type of explant, culture medium used and response.

Despite this long tabulation of reports from a very scattered literature, we still do not know with any high degree of confidence the extent to which one can infallibly rely on any of this "tissue culture" methodology from the perspective of the "clonal fidelity" of Musa plants produced. (In most cases the primary explants have been large. In a few cases substantial chunks of corm tissue are excised. These offer considerable opportunity for cryptic contamination to exist. Just because plantlets are being sold in vitro does not mean they are clean. We have tested enough to know they frequently are not.) Field testing has generally been generally limited, and in some instances, the findings have been ignored, or even seemingly suppressed. Only relatively recently has information begun to emerge from still mostly relatively small scale plantings and trials. The shortcomings of these reports is that one does not know precisely what was "tissue cultured", and more importantly how it was carried out. All too often the people doing the field work are unaware of the details of the tissue culture protocols. On a recent FAO/UNDP consultancy trip to the Indian Institute for Horticultural Research in

Bangalore, I had the opportunity to outline with Dr. R. Dore Swamy (Dore Swamy et al., 1983) a program for correlating tissue culture methodology and propagule/plant performance under field conditions. The results of those tests should be enlightening. My laboratory, finally, has several similar projects in this hemisphere in process using material generated by us.

Aseptic Culture Associated Variation - Somaclonal Variation

I attempted over several years without much success to get large scale plantings of precisely grown, aseptic culture - generated plants field tested. The arguments against large field trials have included the cost, and more significantly, the view has been repeatedly expressed (to my great frustration I might add) that nothing "new" could emerge since the shoot tip or meristem culture techniques were "well established" and "routine." In my view nothing is further from the truth. Perception that the matter was solved, worked against the facts. I blame "industry" and "commercial" laboratory operations for promulgating this view. I suspected from the context of some of our own work on other plants undertaken years ago that the outcome vis a vis stability was concerned might depend on what was done and how it was done. I stated specifically for Musa that this was a question that needed answering (Krikorian and Cronauer, 1983a, 1984b; Cronauer and Krikorian, 1985c). There has been information for quite a few years that plantlets of many species produced via "tissue culture", even shoot tips, are not necessarily true to type. The changes encountered may, in some instances, be rather substantial. Additionally, they seem to vary according to the plant and the exact procedures used. There is very little good, basic, information for any plant of the causes of the variation. Certainly none has been published for Musa.

These changes are frequently "lumped" under the all-inclusive and generally uninformative designation of "somaclonal" variation. See Table 1.

Table 1. Some Possible Causes of Somaclonal Variation

- o Specific karyotype selection from mosaic, chimeric and polysomatic tissues and plants
- o Karyotype changes due to differential response to culture procedures (media composition and/or environment)
 - non-disjunctional aneuploidies in culture
 - mitotic arrest leading to polyploid lines
- o Somatic gene rearrangements or mutations of the karyotype
- o Gene amplification or diminution
- o Virus elimination from selected lines of a culture
- o Somatic gene rearrangements or mutations in organellar genomes
- o Altered nucleo-cytoplasmic interactions resulting in regulation changes
- o Sudden reorganization of the genome by transposable elements
- o Variegated position effects of chromosomal rearrangements (inversions, translocations etc.)

In general, it is supposed that the more organized the initial starting explant, the less the variation. The less organized the cultured starting material, the more the variation. On this view, shoot tips would nominally produce the least, if any, variation; and callus, cell suspension or protoplast procedures would progressively generate the most varied plantlets. Since Musa in particular is a genus that has shown considerable potential for variation and mutation, it seemed reasonable to me long ago that potentially useful variation could emerge from tissue culture. Indeed, as many or more examples of useful variants being grown

on a large scale can be cited for bananas and plantains as perhaps any other vegetatively cultivated plant! No man-generated edible Musa is being grown anywhere other than "curiosity" or "germ plasm collection" scale. Nature has given us all the really useful variants of Musa in existence.

Over the years and as I have attempted to learn more and more about performance of tissue culture-generated bananas and plantains, the situation has vacillated between openness and total secrecy. Some few commercial firms have been rather open; most have been very quiet. The few that I have seen first-hand have been operated by very inexperienced individuals with virtually no supervision. Some firms claim the agronomic results of their products have been excellent with no difficulties whatever. Needless to say, no scientific details are ever provided. Some other firms have been promoting their "wares" with elaborate and elegant brochures. One even provides a "clonal guarantee". In reference to all those nominally proven cases where there is no deviation from clonal stability I should like to make the following statement and the following pleas. First of all, it is noteworthy that a clonally generated plant like banana is retaining its stability throughout in vitro manipulation to a level where one can apparently provide a clonal guarantee. After all, growers know that depending on the clone they grow, there are always "off types" that they "rogue out." If, indeed, there are no "off types," then this is a noteworthy scientific accomplishment that should be shared. Secondly, my plea is that you should try to understand what it is that others might be doing which leads to high levels of "off types." We can safely predict that if we know how to retain stability, and how to foster instability, it might be possible to understand better how to plan a strategy that can give us desired change in one or only a few parameters.

As it stands now, there is high confusion as to the true status of affairs. Are we to believe some firms have the system under perfect control, and others are completely incapable of quality control? Were some firms too anxious to make profit without adequate "baseline" work to guarantee their production was high quality? Were those firms who went more slowly at an advantage? The answer to these questions may never be obtained.

But it is hoped that all the above should finally convince investigators that it is unwise to run before one can walk. Too much unjustified faith in "technology" and insufficient appreciation of our lack of understanding of fundamental biological parameters will, hopefully, cause serious researchers to adopt a more realistic stand when undertaking goal-oriented and practical research projects. Basic researchers should resist at all costs the pressures, real or perceived, to claim that a tissue culture "system" is ready for exploitation "before its time." A world-wide recognition of the fundamental nature of many of the problems plant biotechnologists face although recognized and stated by us years ago (Steward and Krikorian, 1975, 1979) is finally surfacing on a major level and a re-evaluation of directions and strategies on how to meet the challenge is sure to emerge (cf. Cocking, 1986).

In terms of banana and plantain tissue culture research, the need at the moment is to ascertain once and for all whether we can reliably control clonal stability of all clones, especially those of all commercially important genotypes, in vitro for use in those cases where stability is essential, and/or whether useful and agronomically significant variants can be produced and selected out by aseptic shoot tip culture or meristem methods. When all is said and done, the fact that large scale production of clonally stable genotypes from in vitro culture is, in my prediction,

going to be a very minor "blip" in the graph of production statistics. After all, there is not all that great and continuing a need for new plantings of Musa (certainly not at the current turnover levels of a plantation lasting 11 years or so). It is the ability to subject a system to selection "pressures" of various sorts without introducing concomitant undesirable changes that will provide the major advantage of knowing that a given system tends to be stable.

Also, the significance for long term germ-plasm storage in vitro should not be underestimated. Musa is very troublesome to maintain in germ plasm collections and it would be wonderful if we could keep it in storage with full realization that it would retain all its clonal characteristics when "grown out." (This will take a fair amount of work, however.) We also need to have excellent data on detecting the existence of certain viruses like CMV and 'Bunchy Top', and the means needed to eliminate them from multiplying shoot tips. The cucumber mosaic virus which attacks bananas is one that apparently mutates readily and hence it becomes critical to make sure that the serological methods to detect virus are fastidious if we are to claim (cf. Gupta, 1986) they are "virus-free" or "virus-indexed". Cucumber mosaic virus antisera from ornamental crops or certain vegetable crops are known to give "false negatives" when used with Musa. Having seen the devastating effects of 'Bunchy Top' and 'Cucumber Mosaic' virus in the field in the Indian subcontinent, I appreciate very well the need to prevent inadvertant introduction of bunchy top infected material into regions of the world (i.e. the Western hemisphere) where it currently does not exist. One is not sure whether 'Bunchy Top' can be gotten rid of via combined thermotherapy and meristem culture. It will

take a long effort to be sure plants are free of bunchy top (cf. Dale, 1987).

Firm scientific answers to all these questions should have long since been available for Musa, but failure of funding agencies to fund, thus permitting the scientific community to act, misguided secrecy on the part of industry and commercial laboratories, and, a fair amount of propaganda on the part of both communities and the lay and "pseudo-scientific" press (cf. e.g. Anonymous, 1981), all helped to create perceptions that all was well, and, ultimately this all helped to confuse matters. As I put it, we tend to "believe what we want to believe." I also learned to my chagrin that no one wants to listen to a conservative viewpoint when a more attractive, albeit inaccurate, "quick fix" approach is being put forth simultaneously. It is no satisfaction to me to say that I was right. It is also a pity that communications written expressly for the "banana community" have been slow in being published. Even now, one can see in the bibliography a number of "Proceedings" as still "in press," even though the meetings were held long ago.

It may well turn out that we will one day soon be able to "index" plants very accurately and that the aberrant phenotypes observed from Musa tissue culture by some are due to poor 'management' policies in terms of the mechanics of the in vitro culture procedures, and that there is no real basis for concern at all when certain precautions are taken by good investigators. On the other hand, the "somaclonal variation scare" encountered especially seriously in 'Grand Nain', 'Williams' and other AAA clones is real for I have seen it with my own eyes. But the problem may be a blessing in disguise, for there now is a new awareness and this is always good for basic science. Perhaps somaclonal variation, if it is real, can

be put to good use. Perhaps not. We don't know. The important point in all of this to me is that plant tissue culture should not get a 'bad name' because of ignorance, incompetence, and greed.

The encouraging but still very preliminary results obtained by S. C. Hwang and his colleagues in Taiwan, at the Banana Research Institute, Pingtung, with 'Cavendish' clones is a case in point on behalf of the possible potential for useful variant selection. By observing field performance of shoot tip culture-generated plants, they encountered plants with varying degrees of tolerance to Panama Disease or Fusarium wilt race 4. This is but one example of the possibilities for generating disease-tolerant clones (Hwang, 1985). But even here we immediately run into a problem. There is a published report that the shoot tip multiplication system as used in Taiwan is nominally capable of producing high level clonal stability in 'Cavendish' clones (Hwang, Chen, Lin and Lin, 1984). When I first heard of the somaclonal variation encountered in Taiwan as to tolerance against Race 4 Fusarium wilt, I had trouble reconciling this with the contents of the above cited paper from the same Taiwan group reporting stability of type. Ever now, from my perspective, this discrepancy has not been adequately resolved. Also, it is important not to put too much hope in somaclonal variation for disease resistance. Pathogenesis, tolerance, and resistance are complex and poorly understood phenomena and it remains to be shown that the pathogens (especially the leaf-spot ones) for Musa have mechanisms of action which lend themselves to integration with tissue culture selection strategies etc. Many somaclonal "mutants", are not stable, and hence not "real" mutants.

The frustrations and disappointments experienced over the poor performance from a clonal perspective of substantial 'Grand Nain' (AAA)

banana plantings in Jamaica using tissue culture-derived plants has gone far to raise important and obvious additional questions (cf. Oglesby and Griffis, 1986 for their experience, but also Pool and Irizarry, 1986, for their experience in Puerto Rico.) It is also quite clear that off-types can occur in plantains as well (cf. Ramcharan et al., 1987; Vuylsteke and Wilson, 1986). Some even feel that there is a legal aspect to all this.

Conclusions and Commentary

All the above indicates that we must appraise, critically, the existing specifics of Musa tissue culture in great detail, even as we attempt to develop new, and more advanced methods such as mutation breeding, cell and protoplast culture. One can see it either as a dilemma or a challenge. If the research work which I, and many others, feel is important to pursue is to go forward, and if the proper base for scientific work is to be firmly established, this must be done. Similarly, whether one likes it or not, we must face up to the fact that valuable time has been lost due to poor judgement on the part of some, and promotional propaganda on the part of others. My sincere hope is that we can put all that behind us and now do what needs to be done to capitalize on the potential to produce improved bananas and plantains. Would-be supporters of the efforts should not project success at the outset. We need more work and data!

A new awareness of the need for co-ordination of research efforts in attempted improvement of bananas and plantains has emerged over the last few years. INIBAP (International Network for the Improvement of Banana and Plantain) headquartered in Montpellier, France, where a fair amount of research work is being carried out (cf. Tezenas du Montcel, 1987) is attempting to play that role. A major benefit that I see from such efforts

will be that it will be able to encourage focussing of Musa tissue culture work on both scientifically significant and practical problems. Since INIBAP, as an organizing body, has, as yet, no major funds for research, my interpretation is that it will primarily be "a paper and communicative agency." But communication is very important, of course, and if INIBAP does nothing more than foster better information transfer, it will have justified itself. I just hope it does so in a cost effective way. Latin American tissue culture workers interested in Musa, few as they are, through their regional INIBAP directorate at CATIE (Centro Agronomico Tropical de Investigacion y Ensenanza) in Turrialba, Costa Rica and through UPEB--Union de Paises Exportadores de Banano--in Panama ty (UPEB, 1986), and A.C.O.R.B.A.T. (Asociacion para la Cooperacion en Investigaciones Bananeras en el Caribe y en America Tropical), will hopefully be in a particularly strong position to see that their efforts are significant and effectively focussed. The problems here which is all too familiar is that there are insufficient numbers of skilled and experienced researchers in the tropics to take on the work. Also, where there are interested and able people, they are confronted with formidable obstacles. I characterize these problems under the rubric of "If Mother Nature doesn't 'get you', Human Nature will." My point is made and need not be belabored.

Investigators, whoever they are and wherever they may be, should not miss any opportunity to do some good work. The problem will be in the case of INIBAP, indeed any organization seeking to coordinate and stimulate research, that resources will tend to be limited and erratic as to availability, and hence research will be "at the mercy," so to speak, the vicissitudes of attitudes of would-be supporters---government or otherwise. The banana industries, it seems, are unwilling at this point to

support basic research. Long ago a system of surcharges or taxes should have been initiated to glean resources from export profits and thus generate enough money to do significant research. Although there has been some talk in the pages of Informe Mensual of this kind of "revenue enhancement", it will take a considerable change in attitudes before this sort of effort even begins to materialize. Federal granting agencies in the U.S.A. have done a little to help but their budgets are being cut drastically and I am not optimistic that the situation will change. Agencies seemingly always need to appear as if they are "saving the world" with their "new initiatives." Banana and plantain research simply does not, it seems, merit a special place in their list of priorities. Unless a renewed and continuing case is made by political leaders of developing countries that they see banana and plantain research as an unending high priority, there is little hope that initiatives like INIBAP will realize their full potential. A minimum financial security must be achievable to allow for the needed continuity in research. No amount of talk or holding of meetings will take the place of good research. And, if funds need to be allocated either to research or to administration, you can rest assured that I would vote for research. How those in positions of responsibility would feel need not be conjectured on here. Researchers and administrators know what their respective answer would be!

In any case, from my point of view, the main task ahead for tissue culturists is to test on an on-going basis, under field conditions, a well-conceived series of plantings derived from precisely executed and monitored tissue culture manipulations. If one does not acknowledge that work at this stage is experimental, one is at great risk of being misunderstood by growers and other practical people as to the state of



knowledge (or lack thereof) behind any tissue culture scheme. Even if we were able to predict the field performance of each and every clone, and we are not, we would have to know much more about the economics etc. (Soto, 1985). It seems to me that this is a major role that tissue culture workers in the tropics and subtropics could play for their countries. In short, they should really put the whole matter of Musa tissue culture for practical purposes on a firm base. The scientific challenge all of this poses should not be underestimated. If I were closer to the growing areas, I personally, would take this up as a primary objective. As it is, we are trying to do some of this. I have presented, on several occasions (cf. Krikorian and Cronauer, 1983) a broad list of tasks to be carried out. They still need to be worked on.

Our main hope at Stony Brook is to be able to continue to work on Musa as an experimental system. Cell culture, protoplast culture, embryogenesis, androgenesis, and gynogenesis are all very interesting areas and will be pursued (Krikorian, 1987a,b,c,d), but they will not lead to a new 'miracle banana' or 'miracle plantain' overnight. It is foolish to suggest they will. The last thing I need at Stony Brook is to have it said in the tropics and subtropics that we are working to produce a 'Black Sigatoka'--resistant clone(s) of Musa. One must recognize and more importantly behave as if we believe that bananas and plantains are very important crops and that we need to learn as much about them as we can from all perspectives--this includes how they respond to in vitro methods. For this reason alone, one is able to justify aseptic culture work on Musa.

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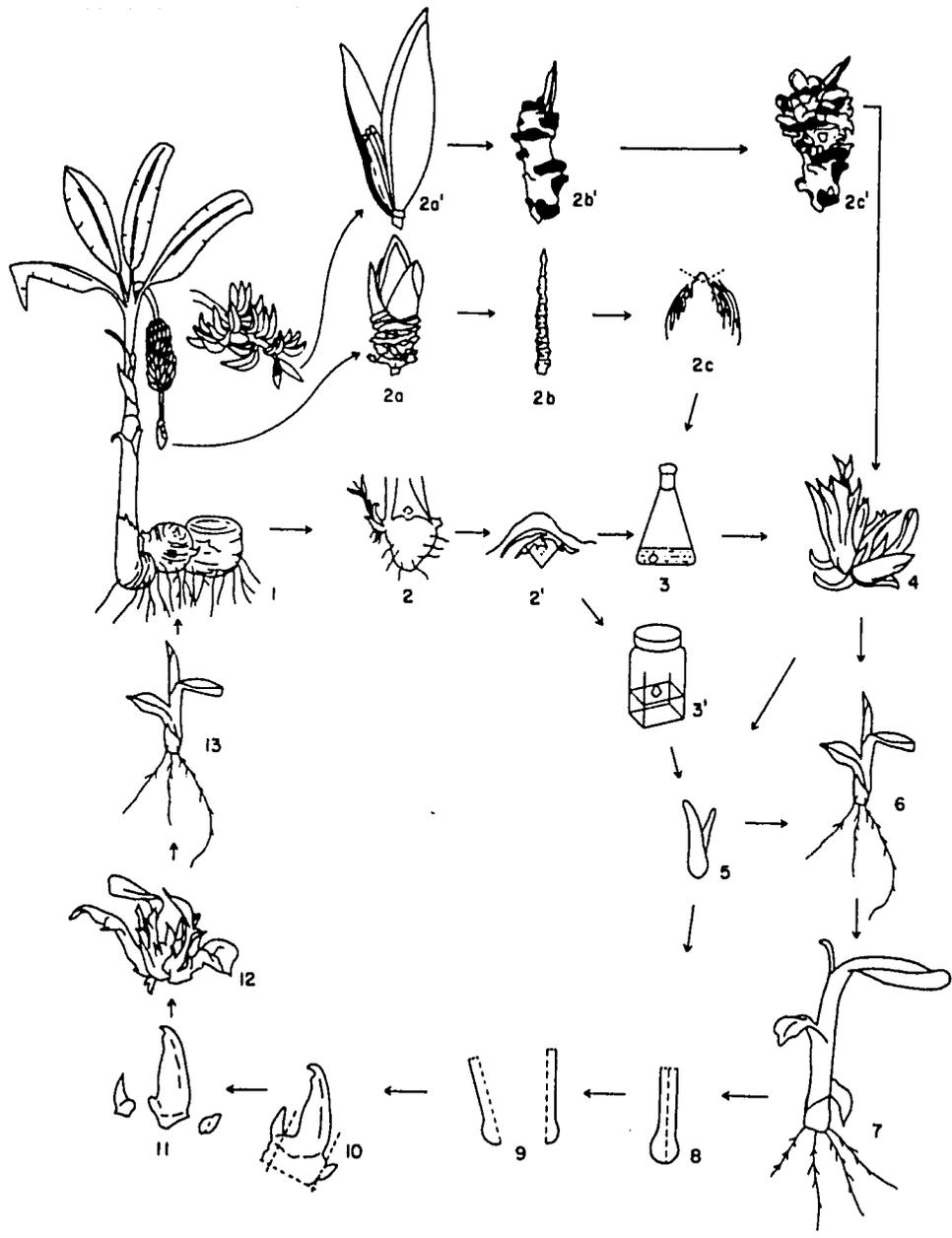
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Figure 1. Diagrammatic representation of multiplication via vegetative shoot tips or meristems (1,2 et seq.), or via floral buds (2a,b,c; 2a',b',c'). By the time multiple shoot formation has been achieved (4), the shoots can be separated (5) and rooted (6,7), or, separated and induced to multiply and initiate new shoots (8,9,10,11,12).



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Callus and Cell Culture, Somatic
Embryogenesis, Androgenesis and Related Techniques
for Musa Improvement

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Introduction

Plant cell, tissue and organ culture is a frontier area of biotechnology which is gaining in importance. Research carried out in most countries has primarily been academic, but in many places it has been recognized for a number of years that a great potential exists for utilizing aseptic culture and related techniques in the improvement and management of horticultural and agricultural crops. The techniques of tissue and cell culture that can be utilized most profitably fall into the following broad areas: 1) rapid and massive clonal multiplications; 2) production of haploids or homozygous diploids and triploids through ovule, anther, pollen and endosperm culture respectively; 3) embryo culture for rescuing progeny of difficult crosses and; 4) generation of "somaclones" or tissue culture-associated variants.

A major constraint to the effective formulation of research and development initiatives in less economically developed and developed places alike, has been, in part, failure to appreciate fully the limitations as well as the capabilities of the available methodologies. In defense of all concerned, it should be stated that the "new biotechnologies" have until only recently been naively heralded by many, virtually everywhere, as offering the ultimate guarantee of realizing broad and sweeping solutions to the world's agricultural and plant biological problems. Like other evolving methodologies and trends, it is often difficult to separate possibility from accomplishment and reasonable expectation from fantasy. There is no doubt that aseptic culture and molecular biology methods offer powerful tools for investigation, and in some cases for development, but before we can expect routine implementation at the practical level, we need to learn a great deal more about fundamental physiological and cellular

processes of in vitro systems. This conservative view of Musa improvement and biotechnology is justified and continues the stance taken in earlier statements on the potential role of tissue culture and the genetic engineering of bananas and plantains and indeed, virtually all plants (Krikorian, 1982 and in press; Simmonds, 1983; Krikorian and Cronauer, 1984 a,b).

Morphogenetic Competence and Totipotency

Most experiments in vitro on the apical growing regions of Musa have been aimed at showing potentialities of the shoot tip, apical meristems with or without leaf primordia or subjacent tissues, and their growth and development under particular conditions. As a result of this work, the means whereby select germ plasm can be rapidly multiplied in a specific-pathogen-free state is now more or less worked out. Shoot tips comprising a few or several leaf primordia or, with greater difficulty even strict apical meristems, can be excised under aseptic conditions, induced to proliferate and form multiple shoots which can, in turn, be separated mechanically and rooted at a high level of efficiency (cf. e.g. Krikorian and Cronauer, 1984a; Vuylsteke and De Langhe, 1985; Cronauer and Krikorian, 1985a and 1986b and references there cited). These plants can be reared to maturity ex vitro. The apex of that part of the inflorescence bearing the male flowers of what we call indeterminate clones - that is clones which have, in theory at least, the morphological capacity for continued elaboration of primordia on the flanks of their floral apical meristems - can similarly be excised and stimulated in vitro to re-initiate growth and form vegetative leaf primordia and shoots that behave as if they were derived from apices of non-flowering corms or suckers (Cronauer and Krikorian, 1985b). While the morphological origin of the response differs,

terminal growing point axes of male flower buds from Musa clones which are determinate as to their growth mode, can also give rise to vegetative shoots. If intervention by in vitro techniques is not implemented in this kind of male bud axis, it is a morphological impossibility, as it were, to form additional bract or leaf primordia and buds on the "terminal" structure (Cronauer and Krikorian, 1986a and in process). The dessert bananas and the 'French type' plantains and cooking bananas are, on the criteria given above, of the indeterminate male bud types; the 'False or True Horn' plantains would be categorized by us as determinate (cf. Tezenas du Montcel et al., 1983). All this work underscores the full developmental potential of shoot apical tissues of Musa and their competence to yield complete plants.

Despite this technical capacity, we still do not know the extent to which one can infallibly rely on the in vitro methodology that flows from the above in terms of clonal fidelity of Musa plants produced from meristem or shoot tip culture. There is confusion as to whether plants multiplied by these means are clonally stable, or whether they show varying amounts of variability. The finding of tissue culture-associated variation among bananas and plantains reared via shoot tip culture (Pool and Irizarry, in press; Ramcharan et al., in press; Vuylsteke and Wilson, 1986), early recognized by me as a possibility, has made it all the more urgent to determine whether one can delineate in the first instance, and ultimately to understand the basis of, methods whereby aseptic shoot and tissue culture systems can be used as a vehicle to achieve production with strictly clonal or true-to type fidelity on the one hand, or to generate useful change in on the other.

The question arises whether or not shoots can develop in Musa from one or only a few cells under certain circumstances - including in vitro culture. Adventitious meristems can arise from a single cell in some plant species. While some shoots arise from pre-existing buds in the in vitro multiplying Musa shoot systems, adventitious buds can, and do, form in large numbers on multiplying shoot complexes. It is not yet known whether any originate from single cells (Banerjee et al., 1986; Cronauer and Krikorian, 1987).

This means that at present, and until shown otherwise, one seems to be dependent on shoots of multi-cell origin (cf. Stewart and Dermen, 1979). The implications of this for generation of variants or mutants from aseptically cultured shoots of Musa will become apparent (cf. DeGuzman et al., 1978; Menendez and Loor, 1979; Novak et al., in press). In tissue culture, it is generally supposed that the more organized the starting explant, the less the variation in a culture-generated propagule. The less organized the cultured starting material, the more the variation. On this view, meristems and shoot tips would generate less variation, and callus, cell suspension or protoplast procedures would generate the most varied plantlets.

In the majority of cases that I can think of at present, genetic engineering procedures such as gene insertion will, perforce, depend on successful regeneration of a plant from cells and protoplasts. Even procedures involving pollen genome modification will benefit from such capability and screening embryoids from pollen will require androgenesis to be achievable at a reasonably high efficiency (cf. Knox et al., 1986). In short, in my opinion, all prospective advantages that might accrue to a banana-plantain breeding or improvement program, other than the obvious

ones such as embryo rescue and multiplication via meristems and shoot tips, demand availability of dependable cell, protoplast, anther-pollen-ovule culture procedures (cf. Krikorian and Cronauer, 1984b).

Studies on Callus and Cell Culture

This laboratory embarked upon a broadly based tissue culture of Musa effort because of our interest in the problems of growth and development, and our expertise in aseptic culture methodologies. A long standing interest in monocotyledons, especially perennial ones, and early exposure to tissue and cell culture of banana, first of fruit, at Cornell University while I was a student in F. C. Steward's laboratory, and then through close friendship with the now late Emerita de Guzman of the University of the Philippines at Los Baños, with shoot tip methods, provided additional bases of incentive. We knew at the outset of our studies that it would not be an easy undertaking. Our successes with daylily (cf. Krikorian et al., 1986) and oil palm (cf. Krikorian and Kann, 1986), however, served to encourage us. Most of the available literature on Musa, such as was then available, was not detailed enough to be very helpful. The work of Mohan Ram and Steward, essentially terminated in 1961, still provided us with the best leads for callus and cell culture work (Mohan Ram and Steward, 1964). We were able essentially to repeat the fruit callus and cell culture work but healthy cultures from virtually all morphological origins which were readily sustainable as to vigor were very difficult to obtain. Cells in suspension were almost inevitably of the "wrong kind" and lacked those qualities that experienced investigators identify as of high likelihood to be morphogenetically competent. Darkening and oxidation was frequently a problem. A frank assessment of our ability to initiate and reliably sustain cultures of either bananas or plantains using "updated know-how"

led to the conclusion that Musa truly seemed to be an extremely recalcitrant system (cf. also Bakry et al., 1985).

In the course of our work Sandra S. Cronauer, then a doctoral candidate in my laboratory (and now a Research Associate), was able to initiate fine, healthy cell suspensions from which globular or proembryonic structures were derived. The structures could be obtained in relatively large numbers from the cooking bananas (ABB) 'Saba' (actually later found out to be more accurately called 'Cardaba') and 'Pelipita'. So-called 'Horn Plantain', ('Platano Commun' or 'Harton' or 'Platano Cuerno', AAB) also responded. Although it was not possible to report the development of a well-formed shoot apex from the structures, we interpreted them as somatic embryos based on their gross morphology and great similarity to embryos of seeded members of the Scitaminae (Cronauer and Krikorian, 1983). We did histology on this material as well. Since a number of embryo mutants exist which are unable to form leaf primordia (cf. Caruso, 1968; Sheridan and Neuffer, 1982), we were not then preoccupied with the failure of our somatic embryos to develop shoots. Since Musa has generally proven itself as difficult to work with from the perspective of callus and suspensions, we saw and still see, this work as an advance. Also, 'neomorphs,' or embryonal structures which reflect aberrations and deviations or alternative pathways in development, had been encountered in daylilies and oil palm and could be controllably re-programmed to yield plantlets (cf. Krikorian and Kann, 1981). Rowe and Richardson (1975) show several zygotic embryos of Musa grown in vitro that are very suggestive of some of our "germinated" somatic embryos, or perhaps more precisely, neomorphs.

Since then, additional efforts have been placed on re-assessing our strategies and further probing the responsiveness of some clones to various modifications to our existing procedures for initiating competent cultures and inducing organized growths. Our outlook continues to be that Musa will, eventually, fully respond in a controlled way.

The general plan of work has been:-

- 1) to concentrate on meristem work with plantains and cooking bananas since this has been the focal point of our financial support. But, as time and resources have permitted we have tried:-
- 2) to induce active growth and cell proliferation from cells as they exist in situ from starting materials which derive from multiplying shoots and a wide range of growing areas of plantlets which, preferably, are aseptically derived by vegetative and floral meristem culture. Also, we try to use explants from mature and young plants maintained by us either in vitro or ex vitro or such as may be made available to us from time to time through the cooperation of others.
- 3) to obtain, maintain and multiply in liquid and semi-solid culture relatively large units and masses, cell clusters and even free cells, the behavior of which can then be compared and contrasted as they develop when transferred or subcultured into fresh medium (or distributed into or onto agar media) into unorganized masses or callus, on the one hand, and into organized structures on the other.
- 4) to investigate the best physical and exogenous chemical environments which promote organized growth or even somatic embryogenesis to proceed from large or small units, clumps or even single units.

5) to examine the relationship between morphological origin of starting material, physiological status of primary explant, genotype and capability for organized response.

The investigative procedures being used, by the nature of the problem and by the emphasis upon interactions between factors, are necessarily different from those in which experimental variables at any one time can be reduced to one. The number of cultures in a given experiment needs to be large to comprehend the range of interactions upon which evidence is sought. Where possible, trials are laid out in Latin squares with blocks of treatments. In tissue culture work, research represents a sequence of a series of individual experiments, each one of which is designed to study one or more specific factors--such factors as medium composition, growth regulator type and concentration often interact with the environment and it is very helpful to visualize subtle sensitivities. This permits one to base better the design of a new experiment on the results obtained from the previous one. In this laboratory, photography is used to a substantial degree as a supplement to the more conventional recording of data.

At this point, the systems are so labor-intensive to maintain that it is difficult to work with very many clones at a given time. Moreover, it has not seemed worthwhile to attempt quantification of growth responses. Indeed, we predict that if meaningful quantitative data are readily obtainable, many of the problems which we seek now to solve, will have been solved.

The more recent significant findings from our efforts are:

1) Nodular masses obtained from some ABB cooking bananas ('Cardaba' [of the 'Saba' group], 'Chato' ['Bluggoe'], 'Pelipita', can be serially subcultured at 3 week intervals and maintained on semi-solid

medium and in liquid culture in the presence of cytokinins like N⁶-benzylaminopurine (BAP).

2) These largish (order of 5-10 mm diameter) nodular masses are competent, replete with peripheral growing zones, and readily give rise to shoots either in liquid or on semi-solid media, and from these, rooted plantlets can be produced.

3) With added synthetic auxins such as 2,4-5 trichlorophenoxyacetic acid (2,4,5-T), or 2,4-dichlorophenoxyacetic acid (2,4-D) these nodular masses can be serially subcultured and maintained in liquid culture in a smaller and more compact growth mode which we call calloid.

4) These compact, calloid masses in the smaller growth mode are also competent.

5) With selection and further exposure to auxins such as 2,4-5 T, small, compact, globular masses (order of a few mm ±) form on these larger calloid masses. Following terminology peculiar to my laboratory at Stony Brook, these compact globular masses are referred to, for convenience, as "nubbins" (cf. Krikorian et al., 1986).

6) These nubbins can be produced and serially maintained in liquid media.

7) The morphogenetic competence of the nubbins is beyond all doubt.

8) Shoots can be generated from these nubbins.

9) Plantlets can be increased from these.

It remains to be seen whether a) we can speed things up, and b) whether further breakdown in unit size of these nubbins along the lines that we have successfully adopted for daylily (Hemerocallis) (Krikorian et al., 1986 and references there cited) can be achieved. Because we are

making progress towards achieving competent cell suspensions, it will perhaps now become superfluous to worry about production of germinable somatic embryos in large numbers as long as we can generate propagules from cells multiplied in liquid. Even so, we continue to work towards this goal for it provides basic research opportunities (Cronauer and Krikorian, in progress).

Figure. 1 shows a sequence of development from nubbins of an aseptically cultured cooking banana, 'Carbaba'. It would serve no useful purpose here to recapitulate the precise procedures necessary to induce these. A manuscript providing full details is in preparation (Krikorian, Scott and Cronauer). What is significant to this report is that we know that the system sought is achievable but it must be reduced, or elevated, if you will, to a level so that efficient cell culture techniques which can have practical potential can emerge. We have not yet been able to address the problems fully due to a shortage of support funds. The finding that liquid-cultured small, globular masses of Musa can yield propagules is an important step. But--the full exploitation of the methods, despite our hopes for their routine application, must await a more complete and basic scientific understanding of not only the system in question, but those involving cells from germ plasm of high priority to breeders or improvers. Studies on the nature of the developmental controls which are brought into play during the culture process and release of morphogenetic competence in banana-plantain cells, and their protoplasts could not only lead to the production of large numbers of plants but could help us achieve the variability and develop the selection procedures that are so necessary in the kind of improvement work envisioned (cf. Krikorian and Cronauer, 1984b; Stover and Buddenhagen, 1986).

The flow of work from the above should be to:

- 1) examine the growth characteristics of the nubbin technique-derived progeny and to establish their behavior at the plantlet/plant level.
- 2) extend methods that suffice for generation of competence from nubbins to cultures of free cells to free protoplasts with the emphasis that in order to be useful, the protoplasts should be capable of organized development.
- 3) determine some of the physiological and biochemical mechanisms involved in the release of competence or totipotency.

Figure. 2 republishes a scheme for possible use of cell, protoplast, and shoot apical meristem techniques for producing novel bananas or plantains (cf. Krikorian and Cronauer, 1984). While the part of the scheme dealing with the protoplasts is the most speculative, in the longer run it is likely to be most productive for generation and selection of variants. This is not a criticism, simply a statement of fact and it is recognized that this part of the work is likely to take a fair amount of effort, and hence time. We learned some time ago to make protoplasts from certain Musa preparations (cf. Cronauer and Krikorian, 1987). Our procedures do not differ drastically from those already published (Bakry, 1984; Chen and Zu, 1985).

I re-emphasize, however, that speculations such as those in our scheme are useless unless a capacity to produce or isolate competent cells and protoplasts and to and regenerate protoplasts in large numbers has been developed. Success is affected by many factors. The entire process can be subdivided into three steps: protoplast isolation, protoplast culture, and plant regeneration. The hope at Stony Brook is that we will have the

opportunity to examine each of these steps in depth and to ascertain the conditions necessary for the successful isolation and regeneration of plantain and banana protoplasts.

Conclusions

- 1) Musa continues to present challenges and opportunities to the tissue culture worker.
- 2) Progress has been substantial but we have a long way to go.
- 3) Generation of shoots and plantlets from competent liquid culture-grown small, compact, globules ('nubbins') from some triploid (ABB) cooking bananas has been achieved.
- 4) Preliminary protocols for protoplast preparation and collection have been developed.
- 5) No reports on successful androgenesis or microspore/pollen culture have appeared so far as I know, and I am aware of no work in that direction.
- 6) Ovule culture studies are just starting (Susana Pons, Panama City with A.D. Krikorian as advisor-consultant).
- 7) We are at the "definition of the problems" stage of research.

Directions for Future Work

- 1) Production of morphogenetically competent globules, 'nubbins', in liquid media must be assessed in a broader range of germ plasm.
- 2) Various parameters to enhance the level and speed of response from each competent unit must be investigated and evaluated.
- 3) Special attention must be given to evaluating potential for competent globule production from material of special interest for improvement and to breeders.

- 4) Efforts must be made to reduce further the unit size of the competent globules used for regeneration, preferably down to the single cell level.
- 5) Studies on protoplasts should be intensified to define better how increased numbers can be produced and collected and, eventually regenerated through reliable protocols, into morphogenetically competent units and plantlets.

Final Comments and Recommendations

- 1) The directions for future work fall completely within the resolutions passed at the 3rd International Conference of IARPCB in Abidjan, Côte d'Ivoire 28-31 May 1985, aimed at fostering in vitro work on bananas and plantains.
- 2) More frequent and in depth communication between breeders, pathologists and tissue culturists is needed to facilitate full understanding and coordination of needs, strategies and objectives.
- 3) A "want" or "wish" list should be produced by breeders and pathologists in conjunction with tissue culture experts in light of the very latest views on breeding, pathology and improvement. This should be circulated to all interested.
- 4) Full access to important germ plasm should be given to all bona fide tissue culture investigators. Limitations should be strictly on the basis of availability and legal considerations such as quarantine etc.
- 5) A tissue culture expert group should meet every two years or some other regular basis in a workshop and prescription clinic format so that a detailed "state of the art" assessment can emerge.
- 6) This working group should be so structured and constituted that membership comprises representation from all those laboratories who

- are actively engaged in in vitro work on Musa from all perspectives except nominally clonal multiplication via shoot tips and meristems.
- 7) Regular and updated reports of progress should emerge from the group and circulated through the INIBAP organization.
 - 8) An active program of education of potential granting agencies should be undertaken by INIBAP, and all others able to bring their influence to bear, towards securing a significant and legitimate place for research support on export, dessert bananas (AAA clones). The policy on the part of some to limit research support to plantains and cooking bananas (AAB and ABB clones), and equating support for research on export dessert bananas as contrary to the spirit of helping the peoples of developing countries must be shown to be unjustified.
 - 9) Finally, the culture methods in the title of this report will undoubtedly demand considerable effort for full practical development.
 - 10) A case can readily be made to justify sustained financial support until that end is achieved.

Acknowledgements

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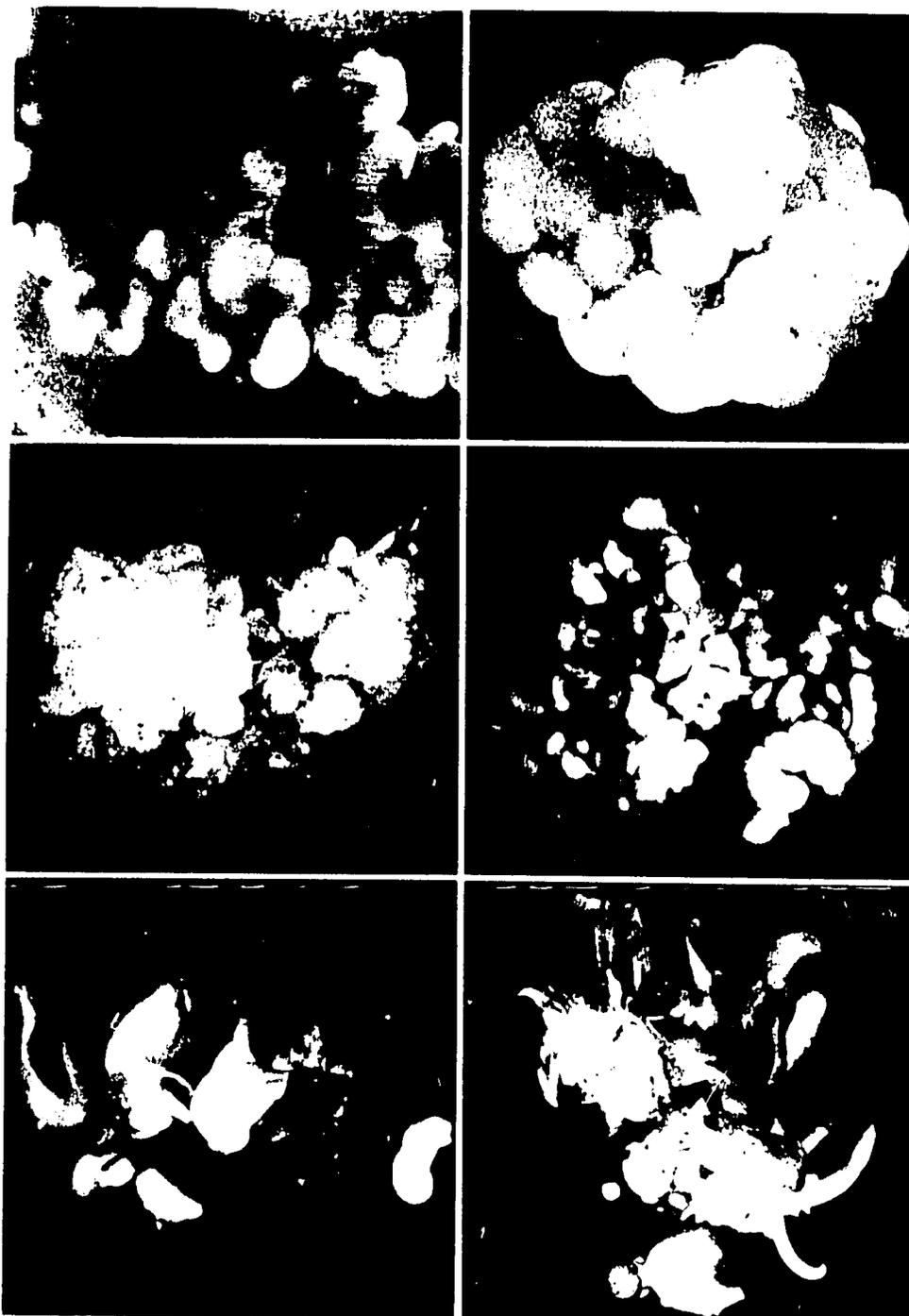
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Fig. 1. Development of shoots of a cooking banana 'Cardaba' (ABB) derived from small, compact globular masses (nubbins) cultured first in liquid media through several stages and then transferred to a semi-solid medium for further shoot growth. A, Close-up of a flask of proliferating nubbins in liquid media, X 2.4; B, close-up of some nubbin clusters generated from a discrete unit and removed from culture vessel for photography, X 9.9; C) nubbin cluster that has further developed and shows organization, X 2.4; D, further organization from a culture similar to that at C, X 2.1; E, emergence of well-formed shoots on semi-solid media, X 2.2; F, proliferating shoot culture, X 2.1. From work of Krikorian and Scott.

Fig. 2. Schemes for possible use of cell, protoplast and apical meristem culture techniques for producing novel plantains or bananas. From Krikorian and Cronauer (1984b).



Regeneration in Bananas and Plantains

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I. INTRODUCTION

Besides being one of man's oldest cultivated crops, edible clones of the genus *Musa*, the bananas and plantains, are among the world's most important crops. In terms of production, they are surpassed only by grapes. In many parts of the world, they constitute a major component of the daily diet in addition to playing an important role as an export crop [Union de Paisés Exportadores de Banano (UPEB), 1983]. Most of the clones of commerce are naturally occurring triploids, are effectively seed sterile, and consequently are propagated vegetatively. The few breeding programs in existence have utilized for some time but only to a limited extent, embryo culture techniques as a means of germplasm rescue (Cox *et al.*, 1960; Rowe and Richardson, 1975). Micropropagation techniques utilizing fairly large shoot tip explants were developed for dessert bananas to produce cucumber mosaic virus-free material and this gradually evolved into a means of ensuring an adequate supply of suitable planting material. Aseptic multiplication of bananas and plantains is now practiced on a fairly wide scale and is on a commercial level in several countries (cf. Cronauer and Krikorian, 1986). These multiplication systems have been improved upon and extended, and can provide uniform material for use in various plant physiological studies and in other aseptic procedures generally included under the broad term of tissue culture.

II. CONVENTIONAL MODES OF MULTIPLICATION

Bananas and plantains are large herbaceous plants which have their center of diversity in Southeast Asia (Simmonds, 1976). The main body of

the plant consists of a large pseudostem, composed of many ensheathing leaf bases which support the large aerial laminae. These structures arise from a massive underground corm which, in the vegetative state, is the true stem with highly compressed internodes. The vegetative apex of the plant lies at the top of the corm at soil level laying down successive leaf primordia. When flowering occurs, the apex becomes much more active as it begins to form flower primordia. A great elongation of the internodes leads to the production of a true aerial stem which is thrust up through the hollow of the pseudostem and eventually bears the fruit bunch.

Because the majority of banana and plantain clones are parthenocarpic and for all practical purposes sterile, multiplication is of necessity by vegetative means. Buds are formed opposed to each ensheathing leaf base of the pseudostem but only a few of these buds ever achieve any size. Young shoots which arise from these buds are called "suckers" and they vary in both size and morphology. Tall, narrow bladed "sword suckers" arise from so-called "button buds" and these may be cut away from the parent plant for use as seed material or allowed to grow and replace the parent plant after a fruit bunch has formed. Shorter, broader bladed "water suckers" are less vigorous than sword suckers and may be trimmed out. Even cut up pieces of corms, so-called "bits," can be used as "seed" material but the most desirable propagules are sword suckers since these produce fruit more quickly than smaller suckers or pieces of corm (Simmonds, 1966).

Unfortunately, reliable quantities of planting material cannot always be obtained by collecting suckers since some clones are known to sucker infrequently or erratically (De Lange, 1969). Barker (1959) showed that additional buds along the surface of the corm could be "released" and stimulated to grow by stripping away some of the outer leaf bases. The small side shoots that develop could be removed from the "mother" plant, and in this way, additional planting material could be obtained. Several other means of stimulating bud development have been reported (Hamilton, 1965; Ascenso, 1967; Turner, 1968; Martinez, 1978) but the stripping method is, by and large, still the most cost effective technique.

III. VALUE OF VARIOUS ASEPTIC CULTURE METHODS

The production of suitable planting material in sufficient quantities can be a troubling task (Krikorian and Cronauer, 1984a). The initiation and establishment of rapidly multiplying aseptic shoot cultures can alleviate this problem since large numbers of plants of a uniform age and size can be

readily generated. This feature can be very attractive in a number of settings. It could remove the variability in size and amount of planting material available, especially for those clones that sucker at erratic rates. In conjunction with a banana breeding program, it would allow the production of increased numbers of the more promising clones so that evaluation could proceed at a more rapid pace. With the spread of the disease "Black Sigatoka" caused by the fungus *Mycosphaerella fijinesis* var. *difformis*, there have been acute shortages of the more highly resistant or tolerant clones such as the plantains "Saba" and "Pelipita" (Stover, 1980; UPEB, 1981; Rowe, 1984). Aseptic multiplication schemes could help to alleviate these shortages. It also could be useful in the rapid distribution of any new disease tolerant or resistant clones which may be produced via conventional breeding techniques (Rowe, 1984). Although a few breeding programs exist, strict quarantine regulations in producer-exporter countries hinder widespread germplasm transfer (Stover, 1977). Theoretically, aseptic shoot cultures could be exchanged across such barriers with a minimum of fuss.

The isolation and culture of shoot tips offer the obvious benefit of the production and multiplication of specific pathogen-free nursery stock and planting material. In 1974, Berg and Bustamante reported the eradication of cucumber mosaic virus from cultured meristem tips using thermotherapy. However, this procedure is not completely effective and careful monitoring must accompany such a procedure to ensure viral elimination. "Bunchy Top" is another virus (or viroid) known to infect bananas, and it is a particular problem in Southeast Asia and even in parts of Africa. However, the effectiveness of meristem culture and thermotherapy for eradicating this agent has not been assessed. Shoot tip culture has proved to be effective in the eradication of "Moko" or bacterial wilt from planting material (Thurston, 1984). And in Taiwan, frequent production of fresh planting material via aseptic shoot tip culture has led to the control of the more virulent races of *Fusarium* wilt in their commercial "Cavendish" banana plantings (Hwang *et al.*, 1984).

IV. MICROPROPAGATION STRATEGIES

A. Culture of Vegetative Apices

Vegetative apices can be isolated from shoots of any size although large shoots are easier to work with. The procedure is begun by peeling away the ensheathing leaf bases of the pseudostem one at a time in a stepwise fashion until the leaf bases become too tiny and delicate to remove by

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hand. Working with the aid of a dissecting microscope, forceps, and scalpel, the final leaf primordia are excised. Since the base of even the smallest leaf primordium will completely encircle the meristem, extreme care must be taken not to damage it. The apical dome is excised by making four angled cuts into the subtending tissue. The size of the explant will vary depending on the size of the shoot from which it is excised.

The excised tissue is surface sterilized for 5 min in a 0.0525% NaOCl solution (1% v/v aqueous dilution of commercial bleach) with a small amount of Tween 20 as a wetting agent. It is then rinsed four times with sterile distilled water and transferred to culture medium. This medium is composed of the mineral salts of Murashige and Skoog (1962), iron chelate of Singh and Krikorian (1980), 100 mg/liter inositol, 5 mg/liter benzylaminopurine (BAP), and 1 mg/liter thiamine-HCl. The pH is adjusted to 5.8 prior to autoclaving. This medium is used as a liquid, 10 ml in a plugged 50-ml Erlenmeyer flask. One apex is inoculated into each flask and they are placed on gyrotary shakers at 80 rpm in an environmental growth chamber maintained at 30°C in a 16 hr daily illumination cycle at 10.2 klx.

Isolated apices green up rapidly and an organized shoot is visible to the naked eye usually within 2 to 3 weeks. Four weeks after isolation, the shoot tip is transferred to fresh liquid medium. After an additional 3 weeks, it is transferred to semisolid medium (0.7% Difco agar) of the same composition. Growth of the shoots and the production of multiple shoot clusters are stimulated by alternating between semisolid and liquid medium at 3-week intervals. The multiple shoot clusters produced are easily subdivided with a few scalpel incisions. Established cultures are maintained on semisolid medium (Fig. 1a) and are routinely subcultured at 3-

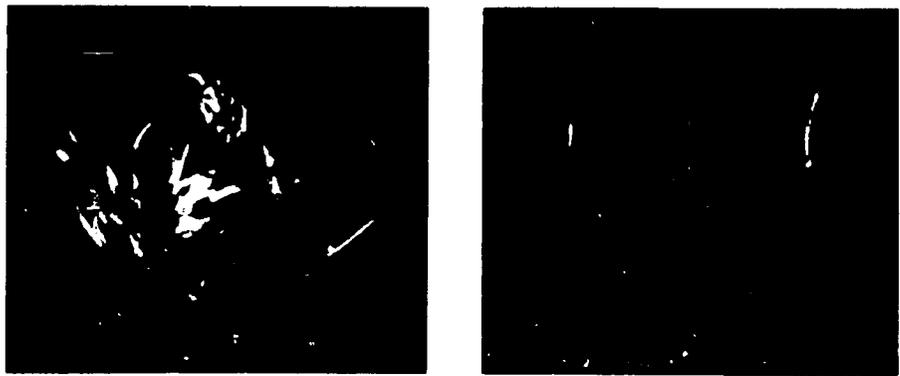


Fig. 1. Multiplication of *Musa* from excised shoot tips. (a) Cluster of proliferated shoots. $\times 0.78$. (b) Plantlets produced by exposure to auxins of shoots separated from clusters such as those in (a). $\times 0.27$.

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week intervals by subdividing the large shoot clusters and placing a few tissue pieces on fresh semisolid medium.

When individual shoots of a culture become large (approximately 6 cm tall by 1 cm diameter), they can be induced to multiply by splitting longitudinally through the apex and placing each half upright in semisolid medium. Small side shoots will be visible within 10 to 14 days and the darkened outer leaves of the original shoot can be cut away. Growth should then proceed as previously described as the shoot clusters increase in size.

Rooted plantlets are produced by placing individual shoots on semisolid medium which contains 1 mg/liter NAA as the sole growth regulator and 0.025% w/v activated charcoal. The first roots usually appear within 3 to 5 days and within 3 weeks root growth is sufficient to support the plantlets when they are potted in a 1:1 peat:vermiculite mix and placed under intermittent mist (Fig. 1b). Plantlets can be transferred to normal greenhouse conditions within 10 to 14 days (for detailed protocols, see Krikorian and Cronauer, 1984b; Cronauer and Krikorian, 1984).

B. Culture of Floral Apices

Aseptic shoot cultures can also be established from floral apices (Cronauer and Krikorian, 1985). In a procedure analogous to that described for vegetative apices, the distal bud is removed from the fruit bunch. In the case of the indeterminate clones, the dessert bananas and the French plantains, successive hands of male flowers and their subtending bracts are removed until they become too small to remove by hand. The removal of the final flower primordia and excision of the apical dome are completed with the aid of a dissecting microscope, forceps, and scalpel. For ease of handling, the apex and a 1-cm block of subtending tissue are excised and surface sterilized as described and the final isolation of the meristematic dome is completed under aseptic conditions (Fig. 2). In the case of the determinate horn plantain clones, successive bracts are removed until the distal flower structures are revealed. The distal 5–6 cm of the inflorescence axis is surface sterilized and cultured in liquid medium. Subsequent culture procedures are identical to those given in Section IV,A.

V. PROBLEMS AND PROSPECTS

It has been convincingly demonstrated by a number of investigators that aseptic shoot tip culture techniques such as the ones outlined here can easily produce thousands of plantlets in a relatively short period of time (Cronauer and Krikorian, 1984; Hwang *et al.*, 1984; Dore Swamy *et al.*,

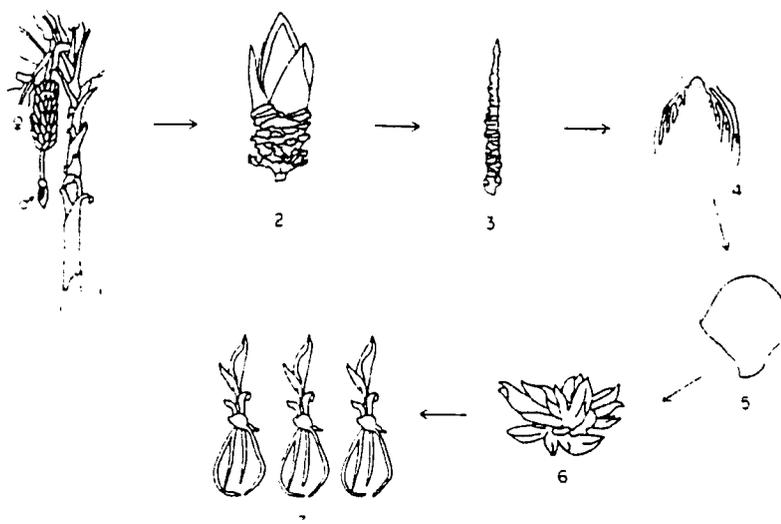


Fig. 2. Diagrammatic representation of the stages of banana floral apex culture. (1) Fruit bunch on a mature plant. The female hands (the fruits) have completely emerged and the apex has continued to produce male flowers. (2) Excised terminal floral bud which is composed of hands of male flower primordia and their large subtending bracts. (3) The same bud as in 2. Most of the flower clusters and bracts have been removed to reveal the peduncle. The apex is still hidden beneath several more layers of flower primordia and bracts. (4) Under a dissecting microscope, the floral apex can be seen to protrude above the smallest flower and bract primordia. The meristem is isolated by making two angled cuts (dotted lines) into the subtending tissue. (5) The excised floral apex is placed into a liquid culture medium of the same composition as given for vegetative apices (see Section IV.A). (6) Multiple shoot clusters arise directly from cultured apices without an intervening callus stage and are maintainable on semisolid medium as described in Section IV.A. (7) Individual shoots can be rooted, potted in a soil mix, and transferred to the greenhouse.

1983). Although the reduced stooling rates of some clones are reflected in the aseptically cultured materials (Cronauer and Krikorian, 1984, and unpublished results) the techniques can still greatly aid in the production of planting material. The application of these techniques in the eradication of diseases such as "Bunchy Top" and "Elephantiasis" needs to be investigated (Stover, 1972; Gomez, 1983). However, before one labels any *in vitro* multiplied clone as "pathogen free" and before it is widely distributed, extreme care must be taken to ensure that one is not unknowingly disseminating diseased material.

Since the breeding of nominally improved, or at least more disease tolerant or resistant clones of bananas and plantains, is such a difficult task, interest has more recently focused on the possibility of inducing variation in cultured material either by the direct application of mutagenic agents or through the recovery of spontaneous mutants or so-called somaclonal vari-

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ants generated by the aseptic culture procedures themselves. Callus tissue has been produced from a number of explant sources (Mohan Ram and Steward, 1964; Srinivasa Rao *et al.*, 1982; Cronauer and Krikorian, 1983), but recovery of plantlets directly from callus tissues is still a challenge. Regeneration of plantlets from the culture of banana protoplasts is sure to be more problematic. This area of research is one on which future efforts need to be concentrated since the potential benefits which could be reaped would be well worth the energies expended (Krikorian and Cronauer, 1984a).

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Temporal, Spatial and Morphological Aspects
of Multiplication in Aseptically
Cultured Musa Clones

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VEGETATIVE APEX CULTURE

Workers in a number of laboratories have shown that vegetative apices of various Musa clones are very responsive to in vitro shoot multiplication techniques (Cronauer and Krikorian, 1984; Hwang, et al., 1984; Dore Swamy, et al., 1983; Mante and Tepper, 1983). In our laboratory, we have worked with 11 economically important triploid clones (4 dessert bananas (AAA), 5 cooking bananas (ABB), and 2 plantains (AAB)) as well as diploids (AA and BB). In a fairly straightforward procedure, the ensheathing leaf bases which surround and protect the apex are removed in step-wise fashion. The shoot tip (meristem plus 1 or 2 leaf primordia) is excised by making 4 angled cuts into the subtending tissue (Fig. 1, steps 1-2') and it is surface sterilized by soaking for 5 min. in a 0.05% NaOCl solution with a small amount of Tween 20 as a wetting agent. Isolated apices are rinsed 4 times with sterile distilled water and cultured on a medium composed of the mineral salts of Murashige and Skoog (1962) supplemented with 100 mg/l inositol, 1 mg/l thiamine HCl, 5 mg/l BAP, and 4% (w/v) sucrose. The pH of the medium is adjusted to 5.8 prior to autoclaving and it can be used either as a liquid or can be solidified with 0.7% Difco agar. Cultures are maintained in environmental growth chambers at 30° C in a 16 hr daily illumination cycle at 10.2 Klux.

Small green shoots, clearly visible to the naked eye, usually form within 21 days. The initial rate of growth and the production of multiple shoot forms are stimulated by the

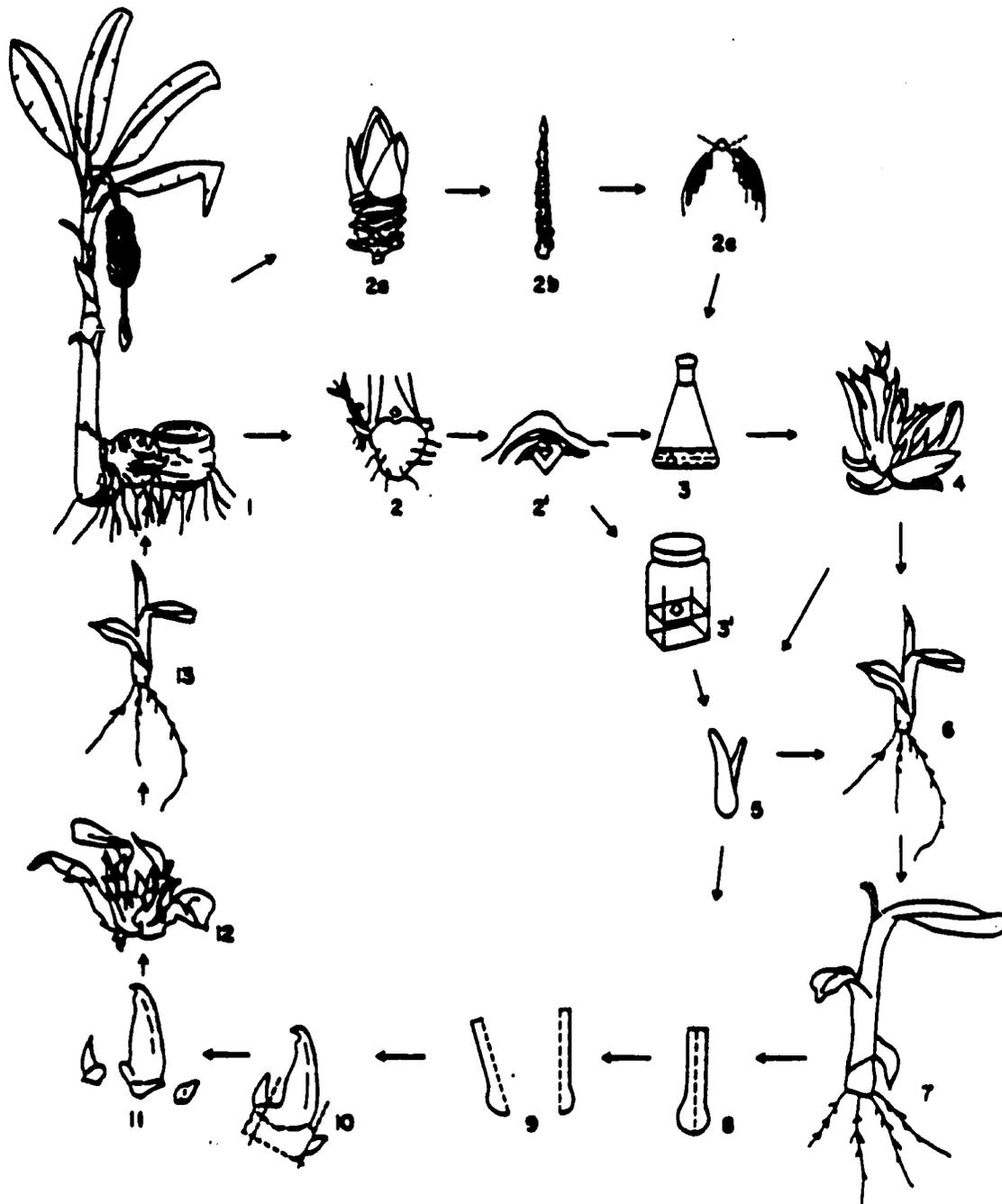


Fig. 1. Diagrammatic representation of *in vitro* multiplication of *Musa* clones using vegetative apices (steps 1-7) or floral apices (steps 1-2c). Buds can be released and individual cultured shoots can be induced to multiply by splitting longitudinally through the apex (steps 8-12). Rooting is readily induced to produce young plantlets (steps 6 and 13).

use of liquid culture medium. In one experiment, apices cultured on semi-solid medium yielded an average of 1 shoot per apex weighing only 20 mg after 42 days in culture while apices cultured in liquid medium produced an average of 6.6

shoots for an average weight of 2.5 g (Cronauer and Krikorian, 1984). Individual shoots can be induced to multiply by splitting longitudinally through the apex. The first small side shoots are usually visible within 10 to 14 days (Fig. 1, steps 8-11). Multiplying shoot clusters are maintained on semi-solid medium and are regularly subcultured at 3 week intervals by subdividing the large clusters with a few scalpel incisions. The resultant smaller shoot clusters are then placed on fresh semi-solid medium.

Although we have successfully cultured isolated vegetative apices of all clones tested, variability in the level of responsiveness has been observed. One factor which affects the ease with which a multiplying shoot culture can be established is the source of the initial explant. Apices isolated from field-grown plants have responded much more slowly than

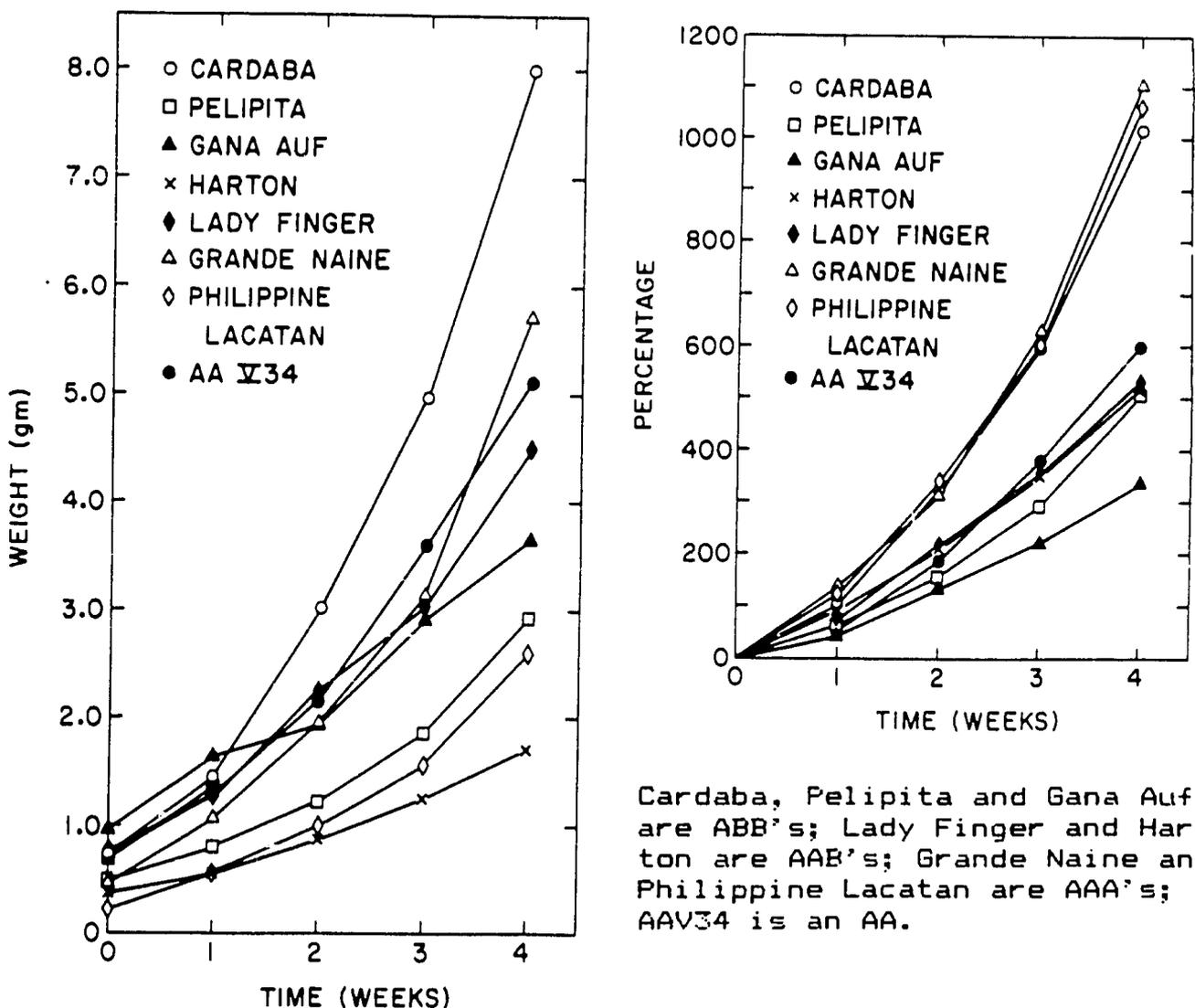


Fig. 2. A. Growth of shoot cultures of 8 Musa clones as reflected by an increase in fresh wt. over a 4 week period. B. The same data showing the increase in fresh wt. expressed as a percentage of original wt.

apices isolated from in vitro cultured shoots despite the fact that apical explants from large corms were usually much larger in size. This could be due to a "conditioning" response of tissue which has been exposed to aseptic culture conditions so that apices isolated from plantlets derived from cultured material respond more readily to an in vitro environment.

Differences in the stooling properties of various clones exist in the field (deLanghe, 1969) and these differences seem to be reflected in the culture vessel. When one monitors growth in terms of the increase in fresh weight over a set time period, clear differences are observed (Fig. 2). The most rapidly multiplying clones are the dessert bananas (AAA) followed by the diploid M. acuminata clone, the plantain 'Harton' (AAB) and the cooking bananas (ABB) with the clone 'Cardaba' being the one exception. However, we have noted that in some cases, the multiplication rate of shoot material can increase over time. For example, shoots of the clone 'Harton' (AAB) underwent an increase in fresh weight of 470% over a 28 day period (Table 1). When this measurement was repeated 1 year later, the increase over the same time period had climbed to 635%. Admittedly, when shoot cultures are routinely subcultured to fresh medium, one selects for the most rapidly multiplying tissues. However, other clones have continued to sucker erratically, even after extended periods of time in culture.

The following provides a rough estimate of the numbers of shoots that can be generated using our multiplication system. One vegetative apex, < 1mm long, if initially cultured in liquid medium for 30 days and then transferred to semi-solid medium yields, after 60 days in culture a large shoot cluster comprised of 30 shoots. If one sixth of the shoots in this cluster are removed and split to induce multiplication, we can safely estimate that each shoot would yield 5 new shoots after 30 days. If the remaining 25 shoots of the cluster are allowed to grow, their number will increase by 3x over the next 30 days. If this pattern of splitting one sixth of the shoots of a cluster and allowing the remaining shoots to grow undisturbed for 30 days is continued, well over four million shoots can be generated in the course of one year. This is a conservative estimate since we have seen a single split shoot

	<u>Days in culture</u>				
	0	7	14	21	28
'HARTON' (AAB)					
Nov 16 - Dec 12, 1983 fresh weight (g)	0.30	0.55	0.90	1.26	1.71
Increase in fresh weight as a % of original wt		83%	200%	320%	470%
Oct 23 - Nov 20, 1984 fresh weight (g)	0.72	1.38	2.41	3.56	5.29
Increase in fresh weight as a % of original wt		92%	235%	394%	635%

TABLE 1. Increase in fresh weight of multiplying shoot cultures of the clone 'Harton' over a 4 week period. Values represent averages of 16 replicates.

yield up to 27 new shoots as opposed to 5. The final number of shoots produced would, of course, vary depending on the stooling properties of the individual clone (cf. Fig. 2).

Propagation of bananas in the field is accomplished by the removal of side shoots or suckers which are used as seed material. These suckers vary in size and morphology. Short, broad bladed "water" suckers produce leaves similar to those of the parent plant while the tall "sword" suckers produce long, narrow leaves. This phenomenon of dimorphic branches has been described (Cook, 1911) and is reflected in the culture vessel where one can observe well formed shoots composed of many ensheathing leaves like water suckers, and other shoots with fewer, narrow leaves, reminiscent of sword suckers (Fig. 3A). In the field, sword suckers are the propagules of choice since they grow much more vigorously and are the first to form fruit. However, in the culture vessel, such differences are not apparent; both shoot types form roots rapidly and develop into sturdy plantlets.

Even though vegetative propagation of banana clones nominally ensures genetic uniformity among the progeny of a particular plant, spontaneous mutants have been isolated in the field. The widely grown clone 'Robusta' (AAA) which is a dwarf mutant of 'Lacatan' is one example, and it has been estimated that many more mutants go unobserved and uncollect-



Fig. 3. A. A cluster of 'Philippine Lacatan' banana shoots displaying dimorphic branch forms. Thin, "water" sucker-type shoots are at the top and narrow leaved "sword" sucker type shoots are at the bottom of the cluster (0.69X). B. A 'Philippine Lacatan' banana plantlet with variegated leaves (1.8X).

ed (Simmonds, 1966). At Stony Brook, clones which have been in continuous culture for over 6 years have never displayed a reduction in their ability to produce roots and give rise to plantlets. We have observed relatively high levels of uniformity, having found one obvious mutant, a variegated plantlet (Fig. 3B), only once. We must emphasize, however, that the vast majority of shoots from our cultures are not allowed to reach a size suitable for the detection of subtle phenotypic or physiological traits. Flowering and fruiting patterns have not been evaluated in the field on any large scale.

The vast numbers of shoots that comprise the multiplying shoot clusters in our cultures have prompted us to question their site of origin. Since each leaf which comprises the pseudostem of the plant has a leaf opposed bud (Fisher, 1978), multiplication of plants in the field can be accomplished by forcing these otherwise dormant buds to sprout (Barker, 1959). However, in the culture vessel, the arrangement of shoots suggests that some of them may have an adventitious origin. In fact, serial sections of shoot material have shown that in some cases, apical "domes" consist of



Fig. 4. A. A cluster of 'Cardaba' shoots (1.5X). B. Longitudinal section of a 'Cardaba' shoot showing three distinct shoot meristems in row like arrangement. (31.5X).

several apices, arranged in rows (Fig. 4). The continued growth of these apices results in the formation of shoot clusters.

FLORAL APEX CULTURE

The responsiveness of banana apices to in vitro multiplication procedures is not limited to vegetative apices. We have shown that floral apices, when isolated and cultured in an analogous procedure, will respond in an almost identical manner (Fig. 1, steps 1-2c) (Cronauer and Krikorian, 1985). Floral primordia will reorganize to yield a vegetative system, giving rise to small vegetative shoot clusters which are indistinguishable from those derived from vegetative apices (Fig. 1, steps 3-5). These shoot clusters can be subdivided and multiplied on semi-solid medium as previously described for vegetative shoots. Individual shoots can be induced to root on semi-solid medium containing the auxin NAA at 1 mg/l with 0.025 % activated charcoal (Fig. 1, steps 6 and 13), and the resultant plants appear identical to those produced through vegetative apex culture.

CALLUS AND PROTOPLAST CULTURE

While the responsiveness of apices in culture has been amply demonstrated, greater difficulties have been encountered with other tissue explants. This is not unlike other monocotyledons where similar difficulties have been reported. Callus has been obtained along the lines described from explanted fruit tissues (Mohan Ram and Steward, 1964), but no organization was observed. Srinivasa Rao, et al. (1982) reported the production of roots from callus derived from cultured peduncle discs, but without concomitant or subsequent shoot formation. In 1983, we reported the production of somatic embryos from proembryonic masses which were derived from shoot tissues of the clones 'Pelipita' and 'Cardaba' (ABB) that had been cultured in a liquid Murashige and Skoog (1962) medium supplemented with 2,4-D or 2,4,5-T and BAP (Cronauer and Krikorian, 1983). Although the overall morphology of the somatic embryos appeared similar to that of zygotic embryos of plants belonging to the same family, they failed to "germinate." Extensive root elongation could be induced by transferral to a basal medium, but shoot growth was arrested.

The isolation and culture of protoplasts and the subsequent regeneration of plantlets has been reported for an ever growing number of species. The development and refinement of such a protocol for the Musa clones would be especially desirable since all of the important clones of commerce are triploid and effectively seed sterile. The regeneration of plantlets from protoplasts could yield new variants through the recovery of spontaneous or induced mutations or via the fusion of protoplasts derived from superior breeding lines (Krikorian and Cronauer, 1984).

Our initial attempts to produce protoplasts from banana tissue employed an enzyme mixture which consisted of the mineral salts and vitamins of Murashige and Skoog (1962), 1% cellulysin, 0.5% macerase and 1% Rhozyme, 0.05% CaCl_2 , 3 mM MES and mannitol (0.3M) and sorbitol (0.3M) as osmotic stabilizers at pH 5.7. When a pectinase was added to the enzyme mixture, yields were improved. Our best collections have been obtained using the mineral salt medium of Potrykus, et

	CLONE		
	AAA	AAB	BB
MS enzyme	+	+	++
MS enzyme with 1% pectinase	++	++	+++
CS enzyme with 1% pectinase	+++	+++	++++

TABLE 2. Comparative yields (indicated by +'s) of protoplasts from sliced aseptic shoot material of 3 Musa clones. MS=salts of Murashige and Skoog (1962); CS=salts of Potrykus, et al. (1977); enzyme=1% cellulysin, 0.5% macerase, 1% Rhozyme.

al. (1977) designated Cereal Salts (CS) (Table 2). No significant difference in yield was seen between the dessert and cooking bananas while better yields have been obtained from the diploid M. Balbisiana (BB). Protoplasts have remained viable in culture for up to 14 days; however, no divisions have been observed. Nevertheless, the potential benefits that could accrue from the successful culture and regeneration of Musa protoplasts ensures that continued efforts will focus on this area of research.

Conclusions

Work done at Stony Brook as well as elsewhere has shown that shoot multiplication systems for the Musa clones can be readily established. Provided a moderate amount of expertise is available to initiate a multiplying culture, one can literally generate millions of plantlets in a relatively short period of time. In addition to ensuring the growing regions of the world with a steady supply of clonal planting material of uniform age and size, field trials of these in vitro multiplied plantlets will no doubt disclose and characterize spontaneous mutants or somaclonal variants which may prove to be useful in breeding and selection programs. This is not to diminish the growing need and importance of the application of the emerging biotechnologies to an ancient clonal crop such as the dessert and cooking bananas. The production of

callus and/or somatic embryos and the subsequent regeneration of plantlets in quantity will greatly contribute to both our understanding of how the plant's genome can be regulated in vitro as well as the production of more desirable clones of bananas and plantains.

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8. Banana¹ (*Musa* spp.)

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1 Introduction

Bananas and plantains² (clones of the genus *Musa*) are among man's oldest and most valued crop plants. They are large, stooling herbs. The wild species *Musa acuminata* and *M. Balbisiana* are diploid ($2n = 22$). Hybridization between them throughout their evolution has resulted in the emergence of cultivars of various ploidy levels and genome formulae. Using some 15 characteristics, Simmonds and Shepherd (1956) categorized many of the world's cultivars in terms of the genomic contributions made by each of the "parental" species. *M. acuminata* is designated genome A and *M. Balbisiana* is designated genome B. Based on this protocol therefore, these two diploid species are designated AA and BB respectively. Other naturally occurring genotypes are AB, AAA, ABB, AAB, ABBB and AAAA (naturally occurring BBB or BBBB have not been found). The predominant clones of commerce are triploid and sterile, producing fleshy, seedless fruits through parthenocarpy (Simmonds 1962, 1966, 1976).

1.1 Importance

The importance of bananas, cooking bananas and plantains to tropical and subtropical economies cannot be overstated. Indeed, in terms of fruit production the crop is surpassed only by grapes. They are inevitably a domestic staple or complementary food and often play a role as a cash or export crop. In addition to being a major source of food in human and animal nutrition, they have a demonstrated importance for tannin, latex and fibre production. Moreover, they are increasingly playing an important role in the manufacture of alcohol (both as an additive to gasoline – "gasohol" and especially beers and spirits). Even banana vinegar is being produced. Apart from these uses, plantains are very important as shade plants for crops such as coffee, cacao etc. In addition to the more usual uses of plantains as a staple food, there is a growing interest in the expanded use of plantains for special food products such as chips, etc. (Palmer 1979).

1.2 Distribution and Area Under Cultivation

Plantains and bananas are grown worldwide. In 1980, the total production is said to have been estimated conservatively at 60 million tons (FAO Production Year

¹ Technically *Musa* does not fall into the category of trees, however, these large perennials look like trees

² Plantain as a name for bananas which are eaten only when cooked, has been widely and loosely used to refer to both the AAB and ABB triploid groups of starchy bananas. According to Rowe (1984), there is now an effort being made to distinguish between these two cooking groups by referring to only the AAB clones as *plantains* and using the term *cooking banana* for the ABB clones. In this chapter, the terms may be used interchangeably

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Book 1980). Some 18 million tons of "bananas" were grown in Africa (Uganda, Rwanda and Zaire the largest producers); 16 million tons were grown in Asia (India, Philippines, Thailand and Indonesia the largest producers); 16 million tons were grown in South America (Brazil, Ecuador and Colombia the largest producers); 9 million tons in Central America (Mexico, Honduras and Panama the largest producers). The export trade in dessert bananas is said to be around 7 million tons. In short, there is hardly a place in the tropics where they are not grown and in large quantities (see Simmonds 1966 for distribution maps of plantain/banana-producing regions of the world). In Colombia alone in the so-called Zona Rural Cafetera (the rural coffee-growing zone), some 160 kg of plantain per person per year are consumed; in the urban coffee-growing zone, the figure is 64 kg and in the urban areas of the nation at large, the amount is estimated at 32 kg (Arbelaez 1983). These kinds of values do not generally enter world statistics, thus obscuring the real importance in terms of diet, and no doubt similar situations exist elsewhere.

1.3 Diseases

Bananas and plantains are subject to a broad range of diseases and pests. Wardlaw (1972) and Stover (1972) may be consulted for details. Following is a listing of the most important disease problems. Panama Wilt or Panama Disease, caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (E. F. Sm.) Synd. & Hans, has, since the late 1950's, virtually eliminated growing of the once very popular AAA dessert banana Gros Michel. Resistant clones of the Cavendish group (Simmonds 1954a and b) were widely used to replace the susceptible Gros Michel but new races of wilt have emerged in Taiwan (Sun et al. 1978), Republic of South Africa (Bower 1982), Philippines and Australia.

Stover (1980) has presented details on leaf spot organisms in Musae. *Mycosphaerella musicola* (the perfect stage of *Cercospora musae*) causes Sigatoka Leaf Spot; *M. fijiensis* var. *difformis* causes Black Sigatoka. The latter is increasingly becoming a major problem and chemical control is very costly. (See UPEB 1981 for an annotated bibliography on Black Sigatoka, Anonymous 1983).

Cucumber Mosaic Virus (CMV) causes infectious chlorosis, heart rot, mosaic and virus sheath rot (Waite 1960, Yot-Dauthy and Bové 1966, Salih and Nas 1974, Peña-Iglesias et al. 1979). Symptoms vary from mild (Stover and Buddenhagen 1976) to serious (Joshi and Joshi 1975). Bunchy Top, although this very destructive disease was reported from the Fiji Islands in 1890, it was not described as a virus disease until much later (MacGee 1953 and refs. cited therein). Even now, the virus has not been isolated, and while there seems to be some varietal resistance (Yang 1970), phytosanitary measures are the only effective means of control (cf. Allen 1978). Bunchy Top is said to be the only *Musa* virus not present in the Western hemisphere. The major vector is the aphid, *Pentalonia nigronervosa* (Granate Sa and Marques 1971).

Nematodes. Although a large number of root and root knot nematodes infecting *Musa* has been described, the two most important are the root burrowing nematode *Radopholus similis* and *Pratylenchus coffeae*. *Radopholus* is by far the more important (Pinochet and Rowe 1979).

Elephantiasis or Big Foot Disease involves swelling and splitting of the pseudostem. Outbreaks have generally been localized (Stover 1972) but elephantiasis has increasingly been encountered in Colombia (Gomez 1983). The causative organism is not known but it might be due to a mycoplasma or mycoplasma-like organism.

Other problems. Moko Disease or bacterial wilt caused by *Pseudomonas solanacearum*, once rather limited in its distribution to Central America and Trinidad (Buddenhagen 1961), is now much more widely distributed (Stover 1972).

1.4 Conventional Propagation and Improvement

Clones of *Musa* which yield edible fruit are propagated vegetatively and grow out of a massive underground corm with highly compressed internodes. In the developing plant, active vegetative growth is localized on the flanks of the broad flattened crown. Growing upward from these areas are the leaf sheaths which encircle each other, and these make up the pseudostem, which in turn, supports the aerial laminae. The central growing point is located at the center of the crown, which remains quiescent during vegetative growth, but becomes more active when flowering occurs. As flowering ensues, the site of active growth in the terminal growing point shifts from the flanks to the central region and elongation takes place. The true stem is pushed up as it develops into a long peduncle (cf. Barker and Steward 1962).

At the sides and around the crown of the corm, the so-called button buds may arise. These may produce young shoots called sword suckers which arise at the base of the main trunk. In agronomic practice, these sword suckers are mainly trimmed out but, every 6 months or so, for each plant, a new sucker is allowed to grow alongside the main pseudostem. After the older shoot has flowered and thus terminated its production of fruit, a new pseudostem is allowed to become the main plant. So-called water suckers are also sometimes produced alongside the main pseudostem, but are less vigorous in growth than the sword suckers (Eastwood 1930, Elayda and Morada 1931, Barnett 1947). Since virtually all the edible clones are parthenocarpic and for all practical purposes seedless or seed-sterile, multiplication is of necessity by vegetative means. Buds, suckers, portions of corms, so-called bits, have all served as "seed" for new plants albeit the most desirable starting point is the sword sucker, since these produce fruit more quickly than the smaller suckers or pieces of corm (Eastwood 1930). Despite the availability of these various propagules, there has frequently been a need for more "seed stock" than is normally available at any given point in time.

As the needs for additional planting material surfaced, various systems for maximum multiplication have been developed. In one such instance, Barker (1959) examined the potential for increased suckering by stripping the older leaf sheaths from the pseudostem, thus permitting buds that never achieve any degree of development under field conditions to develop. Several other means of stimulating bud development have also been reported and assessed (cf. e.g., Hamilton 1965, Ascenso 1967, Turner 1968, Martinez 1978), but the stripping method remains the preferred one, provided some degree of sophisticated management is achievable.

1.5 Banana Breeding

Edible bananas and plantains are perhaps the most conspicuously sterile of all fruit crops, and breeding is fraught with great difficulties (Rowe and Richardson 1975, Rowe 1981, 1984). Crossing using pollen from male-fertile plants and ovaries of male-sterile but female-fertile triploid clones has been the prime strategy (Rowe and Richardson 1975). This yields a miniscule number of tetraploid seeds which are obtained with considerable inconvenience, planted and evaluated. About 3 years are required before one sees the results of a cross. In dessert bananas of export quality, one can appreciate the difficulties of breeding new clones if one contemplates the very strict agronomic and marketing requirements and the very limited female germ line available. For all practical purposes, the Highgate banana is the sole female parent in virtually all breeding work. Attempts to breed-in resistance to such diseases as Black Sigatoka involves using diploid pollen parents originated from wild sources which have resistance. A few organizations have resistant diploids available to them, which they have "synthesized" over a period of years and which are thus ready to be used for "direct incorporation". However, most would-be hybridizers will have to seek resistance in wild diploid sources far from the long breeding sequence designed to end with a commercial tetraploid (Krikorian 1982b). Resistance to important diseases such as *Fusarium* wilt., Sigatoka, Black Sigatoka and resistance to nematodes such as *Radopholus similis* have been by far the main breeding objectives in dessert bananas, but it is fair to say that the problems remain, as no new clone has been introduced to the trade in over 60 years (Rowe 1981, 1984).

There has been much less work on breeding of plantains (DeLanghe 1969, 1976, Rowe 1976, 1984). The needs and problems of banana-plantain improvement would seem to require a continued search for resistant germplasm. Once germplasm with acceptable or desirable qualities insofar as resistance is concerned has been identified, the next step would be to multiply it and distribute it for planting and evaluation in various locations. There has been surprisingly little effort, however, to popularize good clones outside their usual areas of familiarity or distribution. One might have guessed that this would have been a routine strategy in husbanding this crop, but the history of serious research on bananas-plantains has been, in our view, like that of so many other crop plants, one of crisis management.

1.6 Need to Incorporate Unconventional Methods in Banana and Plantain Improvement

Mention has been made of the value of identifying germplasm with desirable qualities such as resistance and distributing it for evaluation (Krikorian 1982b). Far less effort has gone into identifying resistant clones that meet with consumer approval in a given area and to introducing them outside the area of their normal range than might be supposed. In addition to the problem of general unavailability of many of the potentially desirable clones, strict quarantine laws (cf. Stover 1977) frequently hamper or preclude admission of stock even if it were available. Major effects were made by the United Fruit Company in the late 1950's and early 1960's to assemble broad germplasm collections. There still is some interest

throughout the tropics in organizing "open" banks for conservation and breeding, but the problems confronting those who wish to do this are not insignificant. The best and largest collections in the western hemisphere (e.g., those of the United Fruit Company in La Lima, Honduras and the Banana Company of Jamaica, Bodles) are neither completely nor freely accessible and are under commercial control. While many agree that efforts must still be made, not only to expand the disease-resistant germplasm base (Rowe 1984), but to integrate it into an ideal product that combines all the desirable qualities, failures to make freely available the existing Black Sigatoka-resistant synthetic diploids for general breeding efforts is a case in point which emphasizes the nature of the problem. In addition to proprietary prerogative, one frequently has added frustrations of bureaucratic obstacles preventing free importation of germplasm even by responsible investigators and government representatives.

If conventional breeding is one problem, and location, multiplication and distribution of disease-resistant and tolerant clones is another, the feasibility of educating new consumers poses still a third difficulty. A fourth is the problem of convincing responsible organizations that there are good reasons now for developing and perfecting a so-called tissue, cell and protoplast culture technology for *Musa*. Whether it will have a quick "pay-off" cannot be predicted. Since conventional breeding has been shown to be risky, time-consuming and cannot keep pace with the potential development of new virulent races of pathogens, it is imperative that alternative supplemental techniques be found to fight the continuing and seemingly ever-increasing threats to one of our most valuable and popular food sources.

2 In Vitro Approaches

2.1 Significance of In Vitro Techniques

The shoot tip multiplication method obviously has great potential for producing specific pathogen-free planting materials in quantity, and diseases can be eliminated from nursery stock. While ensuing potential re-infection in the field would not be eliminated by this method, starting with clean "suckers" can play a major hygienic role in minimizing problems. The method lends itself readily to getting rid of specific fungal pathogens. For instance, the highly virulent races of *Fusarium* wilt organism ordinarily thought of as innocuous to commercial Cavendish clones (Su et al. 1977), have been dealt with responsibly and with imagination by the Taiwan government. A programme adopted by them to keep *Fusarium* wilt in partial check entails frequent production of fresh planting material with shoot tip culture techniques. The crop is essentially grown as an annual plant. While utilizing aseptically cultured stock as planting material is a labour-intensive device, it has worked well in that island setting. Moreover, harvesting of bananas on a strict schedule for the lucrative export market intended for Japan apparently makes it commercially viable.

Moko or bacterial wilt is yet another example of a specific pathogen that can be easily eliminated from planting material by meristem or shoot tip culture techniques.

From a commercial perspective, the need for rapid clonal multiplication in terms of introducing new clones, was until recently not recognized as a major limitation, since export banana cultivars were seen to change exceedingly slowly and new industries were relatively scarce. Now, however, one sees worldwide the need to provide large amounts of clean planting material of newer clones to start new plantings, or to replace plantations that have been allowed to run down or deteriorate. Although much has been said about the usefulness of the shoot-tip culture method for shipping germplasm throughout the world (Kahn 1979 and references there cited) more than a few problems are associated with it from the perspective of bananas and plantains (Stover 1977). These can however be solved, and bureaucrats need to be "educated". Mention was made that Bunchy Top does not exist in the western hemisphere. Understandably, precautions must be taken to exclude this disease from being introduced inadvertently. There is no evidence that heat therapy can rid banana-plantain stem tips of Bunchy Top. Techniques such as ELISA (Stevens 1982) are of no use, for the virus (viroid) has never been isolated or purified. Double stranded RNA techniques are of potential use, however, and it is imperative that programs be initiated which will focus on the Bunchy Top problem. If this were done, one could begin to assemble new collections of plantains-bananas from the eastern hemisphere for evaluation elsewhere.

Cucumber Mosaic Virus can be eradicated from meristem cultures with heat therapy (Berg and Bustamante 1974). It is essential, however, that careful monitoring be done on such cultures, preferably using electron microscopy, ELISA and bioassay. Since virus can be transmitted through real seed (Gold 1972) there may even be some justification to go through meristem thermotherapy to rid breeding materials of virus.

Musa germplasm collections are currently maintained at relatively great cost. Meristem culture potentially offers a way around the logistic problems of large field collections. Provided genetic stability can be achieved in culture, and the means to slow down growth can be worked out without sacrifice to genetic integrity, the technique would be very attractive.

2.2 Mutation Breeding Potential

Arguments have been made to stimulate adoption of research programmes to assess the feasibility of perfecting a culture technology which will permit the production of variant germplasm for breeding purposes (Krikorian 1982a). The limited female line for breeding dessert bananas has been mentioned. Screening, selection, production and multiplication of cooking plantains and dessert bananas that are resistant or tolerant to Black Sigatoka and other diseases would also be desirable. Investigations would, of course, have to concentrate on the exact parameters for establishment and growth of morphogenetically competent tissue, cell and protoplast cultures and to define the optimal conditions for their subsequent challenge by pathogens, pathogenic extracts and mutagens. A system capable of yielding entire plants from single cells or protoplasts is an essential and crucial feature and without this capability, there would be no possible means of readily producing, and/or selecting, tolerant or resistant germplasm (Krikorian

and Cronauer 1983). Micropropagation via meristems does have substantial potential for producing variability, but one cannot predict at this time that variability will include resistance to Black Sigatoka disease.

2.3 Summary of the Work Done on *Musa* Clones

The majority of culture work which has been done on *Musa* has focussed on the production of aseptic shoot cultures. Such cultures have been established from a number of clones of dessert bananas (AAA) and plantains (AAB and ABB) using meristems and shoot-tips (meristems plus a few attached leaf primordia). Multiplying shoot cultures have been established by culturing a larger initial shoot explant (order of 1 cm³) which contains the apex and cutting this cube of tissue into quarters (de Guzman et al. 1976, Bower 1982, Manicom 1982). By destroying the central growing point, one presumably releases the axillary buds (actually leaf-opposed, Fisher 1978) which surround the apex and would normally fail to develop. In an attempt to maintain a degree of uniformity in the cultured material, we have worked with very small initial explants (meristems with 1 to 2 small leaf primordia at most) and have cultured them to small single shoots 2 to 3 cm tall. At this point, shoot multiplication is induced by splitting the shoot longitudinally through the apex. Such a technique would also be useful for producing large numbers of virus-free plantlets if the initial explant was rendered virus-free, and later induced to produce additional shoots (see Krikorian and Cronauer 1984 for details).

Nearly all of the published work on shoot culture has emphasized production of rooted plantlets, usually by separating the individual shoots, and transferring them to a separate root-inducing medium (see Table 1). However, some workers have cultured the primary explant on an auxin-containing medium and have measured shoot culture success by scoring for concomitant root formation. While it is true that very young cultured shoots can be induced to root, it has been our observation that once root formation has begun, additional shoot formation is suppressed. If one's main goal is the production of large amounts of shoot material, then rooting should be carried out as a separate step. In this way, an open-ended shoot multiplication system can be established (Cronauer and Krikorian 1984 b). The salient points of the shoot multiplication systems pertaining to *Musa* clones that have been published are summarized in Table 1. Other aspects of aseptic culture of these clones are discussed in the appropriate section.

2.4 Methods of Sterilization

The morphology of the banana plant suggests that the apex might well be naturally free of contaminants since it lies in a depression surrounded and protected by many sheathing leaf bases. However, when one considers the considerable effort expended in the execution of meristem culture work, few workers have risked the possible consequences of not treating excised shoot tips with a sterilizing agent. Sterilization of tissue explants (such as shoot tips, embryos from seeded diploids, or young fruits) has been accomplished by washing the tissue with 0.05 to 0.25% solution of sodium hypochlorite or chlorine-saturated water with a

Table 1. Summary of aseptic culture work on *Musa* clones. Note: This table is presented under three separate headings. A, deals with shoot cultures; B, callus cultures; C, embryo culture

Clone	Culture medium		Initial explant	Conditions of incubation	Type of shoot growth	Rooting conditions days from initial explant to rooted plantlets	Reference
	Medium ^a (and % sucrose) % agar	Growth regulators (mg/l)					
<i>A. Shoot cultures</i>							
Dessert Banana (AAA) Cavendish	SM (3%) 0.8% Difco agar	IAA (2) Kin (2)	Decapitated shoot apex	15°C 200 ft. candles	Adventitious buds, some becoming shoots	Sphagnum mos. 150 days	Ma and Shii (1972)
Dessert Banana (AAA) Cavendish	SM (3%) 0.5% Difco agar and liquid	IAA (2) Kin (2)	Decapitated shoot apex	25°C	Plantlet formation from adventitious buds		Ma and Shii (1974)
Dessert Banana (AAA) Cavendish	Knudson (2%) 0.6% agar	CA (1000) CW (10%)	Meristem	27°C Continuous light	One shoot from each meristem	NAA (1) 150 days	Berg and Bustamante (1974)
Dessert Banana (AAA) Bungulan	MS (3%)	Kin (0.1–5.0) CW (15%)	Shoot tip 0.5 cm ²		Single shoots and shoot clusters	NAA (1) (2.5)	de Guzman (1975)
Dessert Banana (AAA) Philippine Lacatan	MS 2% dextrose	BAP (1.25–20.0) Kin (2.5) CW (15%)	Shoot tip 1 cm at base cut into quarters		Single shoots and shoot clusters		de Guzman et al. (1976)
Dessert Banana (AAA) Cavendish, Gros Michel, Plantains (AAB)	MS (3%) 0.6% agar	IAA (1) BAP (0.5)	Meristem cut vertically 7 to 12 times (incised)	29°C 12:12 L:D 150 lx (13.9 ft. candles)	Each shoot yielded 15 to 20 shoots	No medium change	Vessey and Rivera (1981)

Diploid Bananas, Tetraploid Hybrid Bananas							
"Bananas" (unspecified)	MS (3%) 0.7% agar	IAA (2) BAP (5)	Apical tissue 10 × 10 × 10 mm block, cut into quarters	23 °C	Multiple shoots	Charcoal 10 g l ⁻¹ 27 °C	Bower (1982)
"Bananas" (unspecified)	Modified MS (3%) 0.8% agar	Kin. (5) IBA (2) NAA (2)	Single shoot tips	27 °C	Single rooted plants	No medium change 42 days	Bower (1982)
Dessert Banana (AAA) Williams	Modified MS (3%) 0.8% agar	Kin (5) IBA (2) NAA (2) charcoal 5 g l ⁻¹	Shoot tip	27 °C 16:8 L:D	Single rooted shoots	Simultaneous shoot and root growth emphasized 42 days	Bower and Fraser (1982)
Dessert Banana (AAA) Taiwan Cavendish Grande Naine	SM (3%) 0.7% agar	IAA (2) Kin (2)	Shoot area 1.2 cm ³		Adventitious buds	1 g l ⁻¹ char- coal 70 – 91 days	Hwang (1982 un- published)
"Bananas" (unspecified)	SM (3%) 0.7% agar	IAA (2) BAP (5)	Shoot area 15 × 15 × 10 mm cut into quarters	25 °C 16:8 L:D	Buds	1 g l ⁻¹ char- coal, 29 °C Continuous light or 16:8 L:D, 49 days	Manicom (1982)
Plantain (AAB, ABB)	MS (3%) glucose 0.7% agar	IAA (1) BAP (0.5)	Apical area 1 cm ³		Single shoots	5 g l ⁻¹ char- coal, IAA (1)	Alvarez et al. (1982)
Dessert Banana (AAA) Robusta	MS (2%) 0.8% agar	BAP (5) (10) IBA (5) C ₂ H ₅ (15%)	Apical shoot bud	25 °C 950 – 1000 lx (88 – 93 ft. can- dles)	Single rooted plants	No medium change 49 – 56 days	Dore Swamy et al. (1983)

Table 1 (continued)

Clone	Culture medium		Initial explant	Conditions of incubation	Type of shoot growth	Rooting conditions days from initial explant to rooted plant-lets	Reference
	Medium ^a (and % sucrose) % agar	Growth regulators (mg/l)					
Dessert Banana (AAA) Robusta	MS (2%) 0.8% agar	BAP (10) CW (15%) or BAP (5) Kin (2.5) or Kin (2.5), IBA (5) or 2iP (2), CW (15%)	Axillary buds	25 °C 950 – 1000 lx (88 – 93 ft. candles)	Shoot clusters	IBA (5)	Dore Swamy et al. (1983)
Dessert Banana (AAA) Grande Naine Plantain (ABB) Saba	MS (3%) 0.8% agar	BAP followed by BAP and Kin in rotation	Shoot tip 0.5 cm	25 °C 16:8 L:D 100 – 300 ft. candles	Multiple axillary shoots	MS with no hormones 2 g l ⁻¹ charcoal, 180 days	Hennen (personal communication)
Abaca or Manila Hemp (<i>Musa textilis</i>)	Modified MS (3%) 0.5 – 0.8% agar	BAP (10)	Shoot block 5 – 12 mm thick, containing apex cut into slices 2 – 4 mm thick	26 °C 16:8 L:D 1500 – 5500 lx (139 – 511 ft. candles)	Multiple axillary shoots	NAA (0.1 – 1) IBA (2 – 10) 1 – 1.5% sucrose	Mante and Tepper (1983)
Dessert Banana (AAA) Philippine Lacatan Grande Naine Plantain (ABB) Pelipita Cardaba	MS (4%) 0.7% agar and liquid	BAP (5) BAP/Kin Combinations	Shoot tip	30 °C 16:8 L:D 950 ft. candles	Multiple shoot clusters	0.25 – 2.5 g l ⁻¹ charcoal NAA (1) IBA (1) IAA (1) 49 – 56 Days	Cronauer and Krikorian (1984b)

Table 1 (continued)

Clone	Culture medium		Initial explant	Conditions of incubation	Callus formation	Shoot formation	Root formation	Reference
	Medium (and % sucrose)	Growth regulators (mg l ⁻¹)						
<i>B. Callus culture</i>								
Dessert Banana (AAA) Lacatan Robusta Cavendish Tumoc Gros Michel	White's (2%) 0.5% agar and liquid	CW (5 – 10%) 2,4-D BTOA, 2,3,6-TPA IAA, Kin Coumarin	Fruit plug	24.5 °C	Yes and cell suspension	No	No	Mohan Ram and Steward (1964)
Dessert Banana (AAA) Kluai namwa Kluai hom-thong	White's (2%) 0.6% agar	2,4-D (5)	Fruit plug	25 – 29 °C 80 ft. candles	Yes	No	No	Tongdee and Boon-Long (1973)
Dessert Banana (AAA) Bungulan	White's	2,4-D (2.5) Medium or medium and tissue irradiated 0.1, 1.0, 2.0, 4.0 Kr.	Cross-section of fruit including peel and pulp		Yes	No	No	de Guzman (1975)
Dessert Banana (AAA) Robusta	MS (2%) 0.8% agar	2iP (2) 2,4,5-T (0.1 – 10) IBA 2,4-D, NAA, IAA, BTOA, 2,4,5-T and Kin, BAP, 2iP combinations	Discs of inflorescence axis		Yes	No	Yes	Srinivasa Rao et al. (1982)
Dessert Banana	ND (2%)	IAA Kin	Fruit plug		Yes	No	No	Brasil (1982)

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Table 1 (continued)

Clone	Culture medium		Initial explant	Conditions of incubation	Callus formation	Shoot formation	Root formation	Reference
	Medium (and % sucrose)	Growth regulators (mg l ⁻¹)						
(AAA)	0.5% agar	2,4-D IBA NAA coumarin 2iP						
Plantain (ABB) Pelipita Cardaba	MS (2%)	2,4-D (1) BAP (1, 0.1) 2,4,5-T (1) CW (5%)	Multiplying shoot clusters	30 °C 950 ft. candles 16:8 L:D	Yes	No	Somatic embryos with root elongation	Cronauer and Krikorian (1984a)
Clone	Culture medium		Initial explant	Conditions of incubation	Type of shoot growth	Rooting conditions	Reference	
	Medium (and % sucrose) % agar	Growth regulators (mg l ⁻¹)						
<i>C. Embryo culture</i>								
Diploid Banana (BB)	RC (4%) 0.7% agar	None	Excised embryo		Single-rooted shoot	Soft agar 0.5%	Cox et al. (1960)	
Diploid Bananas	Modified Knudson's (4%) 0.525% agar	None	Excised embryo	-	Single-rooted shoots	No medium change	Rowe and Richardson (1975)	

^a SM = Smith and Murashige (1970); Kundson (1946); MS = Murashige and Skoog (1962); RC = Randolph and Cox (1943); modified Knudson (1950); White (1943); ND = Nash and Davies (1972)
Abbreviations: Kin = Kinetin; CW = coconut water; BAP = 6-benzylamino purine; IAA = indole-3-acetic acid; CA = casamino acids; IBA = indole-3-butyric acid; NAA = naphthaleneacetic acid; 2iP = 6- γ , γ -(dimethylallylamino)purine; BTOA = benzothiazole-2-oxyacetic acid; 2,3,6-TPA = 2,3,6-trichlorophenylacetic acid; 2,4-D = 2,4 dichlorophenoxyacetic acid; 2,4,5-T = 2,4,5-trichlorophenoxyacetic acid

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few drops of a wetting agent such as Tween or Teepol. Different investigators have used varying lengths of time. We have found a 5-min sterilizing wash to be both sufficient and equally effective whether working with excised shoot tips, embryos or immature fruits. These materials are then washed several times with sterile distilled water to remove all traces of the sterilizing agent. They are then ready to be manipulated under aseptic conditions or placed directly onto or into culture media.

2.5 Media Composition

In our preliminary studies of shoot culture, we found that *Musa* shoots could be maintained on a number of different media, but as the work progressed we have sought to use the simplest medium that will support robust shoot growth. The isolated shoot-tips and branching cultures can be supported on Murashige and Skoog (1962) basal salts supplemented with 100 mg l^{-1} inositol, 4% sucrose with 1 mg l^{-1} thiamine HCl as the sole vitamin (designated B_{MS}) and 5 mg l^{-1} BAP. A number of workers have used slightly more complex media for shoot culture initiation and maintenance such as that of Smith and Murashige (1970) or they have added coconut water (10 to 15% by volume), casamino acids or yeast extract (1 g l^{-1}) (see Table 1A). We have found these additives to be unnecessary and thus have tried to avoid using undefined medium components. Nearly all investigators have obtained root formation by modifying the medium only slightly, e.g. adding low levels of auxins or small amounts of activated charcoal.

Early work on fruit callus culture was done using a modified White's (1943) medium, but these cultures were not morphogenetically competent. More recent workers, Srinivasa Rao et al. (1982) and Cronauer and Krikorian (1984a) who have obtained some organ formation have used MS salts supplemented with a number of auxins and cytokinins. We have obtained poor and erratic results when culturing diploid embryos excised from seeds on an MS medium. More predictable results have been obtained using Randolph and Cox salts (1943) as reported by Cox et al. (1960). Rowe and Richardson (1975) have reported satisfactory results using a modified Knudson's (1950) medium. We do not wish to imply that the mere selection of the "correct" medium will necessarily ensure successful *in vitro* culture of banana tissues from all clones. However, it is one of many important factors that the investigators must carefully consider. Further details and discussions on the relevance of medium composition to a particular aspect of the tissue culture of *Musa* species will be deferred to the appropriate section.

2.6 Meristem Culture

Meristem culture has been used to eliminate viruses from a number of plant species (see Chap. II). Commercial clones of bananas and plantains may be infected with symptomless viruses (in particular Cucumber Mosaic Virus) and the effects of such infections on fruit yield have been debated. Berg and Bustamante (1974) reported the isolation and culture of meristems from banana plants and assessed the effectiveness of this technique in eliminating CMV from the cultured plants. A number of other investigators have isolated and cultured shoot tips (see Table 1

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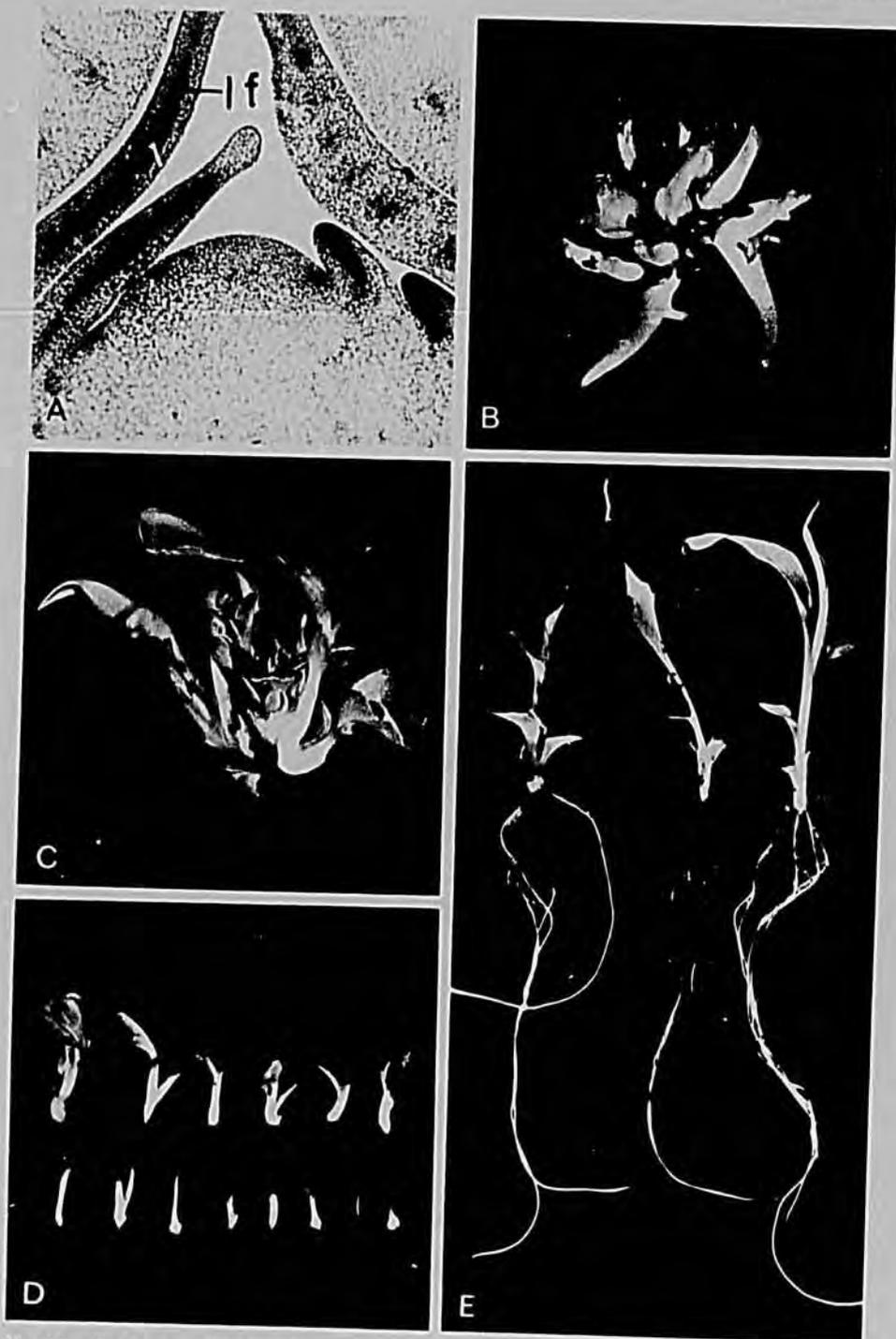


Fig. 1A-E. A Longitudinal section of a desert banana Gros Michel showing the shoot apex and the youngest leaf primordium ($\times 83.5$); B Multiple shoot cluster derived from an excised plantain shoot apex (Cardaba ABB) cultured on $B_{MS} + 5 \text{ mg l}^{-1}$ BAP ($\times 1.6$); C Older Cardaba shoot cluster composed of larger individual shoots ($\times 0.8$); D Individual shoots from the cluster in C, separated by scalpel incisions ($\times 0.4$); E Aseptically rooted Cardaba plantlets ready for transfer to the greenhouse. Roots such as these can be produced in 3 to 4 weeks by placing individual shoots on the maintenance medium supplemented with 0.25% activated charcoal ($\times 0.4$)

and Fig. 1 A). Examination of the illustrations in these reports demonstrates that the size of the initial explant varies widely, ranging from tiny apical explants to large chunks of the shoot tip. Clearly, the smaller the initial explant, the greater the likelihood that any viral contaminant might be eliminated. The majority have used MS medium or as amended by Smith and Murashige (1970) with some additional components (as discussed in media composition section). Isolated meristems, which are initially creamy white, usually begin to turn green within 4 days. The pace at which growth proceeds and the growth form obtained largely depends on the type of culture medium used (liquid or semi-solid). It has been our observation that shoot tips placed in liquid grow faster and produce multiple shoot clusters, while shoot tips cultured on semi-solid medium grow at a slower pace and always yield single shoots (Cronauer and Krikorian 1984 b). Shoot clusters such as in Fig. 1 B can usually be obtained within 6 weeks from apices cultured in liquid. Such a cluster would then be transferred and maintained on semi-solid medium. By subdividing and transferring to fresh semi-solid medium at regular intervals, large number of banana shoots can be obtained from an "open-ended system" (Fig. 1 C and D).

2.7 Embryo Culture

Mention has been made that all of the *Musa* clones of commerce are triploid and seed sterile. However, efforts in improving bananas via conventional breeding techniques rely upon the fact that some "seedless" clones will produce a miniscule number of seeds when pollinated by male diploids. Since the seed germination percentage using conventional techniques is often very low and can be dramatically improved using embryo culture, this technique has become an important method of salvaging the progeny of crosses which would otherwise be unavailable. In 1960, Cox et al. reported the first in vitro culture of *M. balbisiana* embryos. Intact seeds were surface sterilized, and the embryos were cultured on Randolph and Cox (1943) medium with 4% sucrose and 0.5% agar. More recently, Rowe and Richardson (1975) reported that up to 50% germination of excised embryos can be achieved using a modified Knudson's (1950) medium. The germinated embryos develop into young rooted plantlets which can be transferred easily to the soil.

2.8 Establishment of Callus and Suspension Cultures

Although several investigators have attempted to establish callus and subsequently, cell suspensions of bananas, this work has met with very limited success. A number of different tissue explants have been used as the starting material and although some have, from time to time, been able to initiate callus tissue, apparently no shoots or plants have ever been obtained. Mohan Ram and Steward (1964), working with plugs of tissue from immature and mature but preclimacteric fruits, obtained callus-like growths using a number of growth regulators. When these were transferred to liquid medium, slow-growing suspensions were established. However, these cultures proved to be nonmorphogenic. Working with different clones, Tongdee and Boon-Long (1973), and de Guzman (1975) re-

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A



B



C



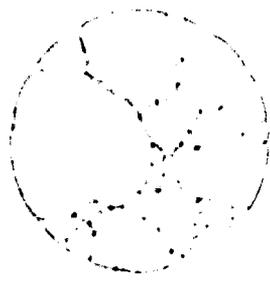
D



E



F



G



H



I

peated some of these studies and obtained callus from fruit explants, but it proved non-transferrable. More recently, Srinivasa Rao et al. (1982) have reported the establishment of callus cultures from discs of the inflorescence axis. This callus could be induced to form roots, but no shoot or bud formation was observed.

In our laboratory, work with fruit explants has confirmed the results discussed above. Tissue plugs placed on medium containing very high levels of auxin and small amounts of activated charcoal produced a white nodular callus. However, such a callus continued to proliferate only through a limited number of transfers (Fig. 2B and C). More recently, we have used aseptically cultured multiplying shoot clusters as a tissue source to establish callus and cell suspensions cultures. Occasionally, when a single shoot is placed on the appropriate semi-solid medium, callus is formed at the shoot base (Fig. 2A). If liquid medium is used instead, the shoot tissue becomes translucent and sloughs off cells. The resulting cell suspensions (Fig. 2E) can be subcultured and they can go on to produce small pieces of nodular callus (Fig. 2D). Under other culture conditions, shoot clusters placed in liquid medium produce small nodular tissue masses which go on to produce somatic embryos (Fig. 3) (Cronauer and Krikorian 1984a). Although the root axis of these embryos can elongate, no shoot growth has occurred and to date plants have not been recovered. The ability of this culture procedure to produce somatic embryos which will yield rooted plantlets is currently being assessed.

2.9 Isolation, Fusion and Culture of Protoplasts

There are no reports in the literature on the isolation and culture of protoplasts from any *Musa* clone. We have developed a procedure which can reproducibly yield protoplasts from multiplying shoot cultures. Aseptic shoot cultures were used as a source material since this eliminates the need to surface sterilize starting material. A cellulase-pectinase mixture is used to produce protoplasts that are fairly uniform in size and appearance (Fig. 2F and G).

Intact cells from several plant species have been mechanically isolated and cultured to produce cell suspensions and callus (Schwenk 1981). We have been able to obtain large numbers of palisade cells from mature banana leaves by incubating the tissue in an enzyme solution which digests the middle lamella and allows the individual cells to break free (Fig. 2H and I).

←
Fig. 2A–I. **A** Callus formation at the base of a young dessert banana shoot (Philippine Lacatan AAA) cultured on Anderson's (1978) salts and 2 mg l^{-1} 2,4,5-T ($\times 1.5$); **B** and **C** Two examples of callus derived from tissue of green, preclimacteric dessert bananas (Cavendish AAA) cultured on B_{MS} salts and 50 mg l^{-1} NAA and 0.25% activated charcoal. **B** $\times 2.4$; **C** $\times 1.7$; **D** Callus pieces produced from cells derived from multiplying shoot cultures of the plantain Pelipita ABB and cultured in liquid medium composed of $1/2 B_{MS} + 2 \text{ mg l}^{-1}$ 2,4-D and 1 mg l^{-1} BAP ($\times 4.7$); **E** A cluster of Pelipita cells grown in suspension in $B_{MS} + 1 \text{ mg l}^{-1}$ 2,4-D and 1 mg l^{-1} BAP ($\times 61.4$); **F** Protoplasts from shoot material of Philippine Lacatan. The enzyme mixture was composed of the salts of Potrykus et al. (1977), 1% Cellulysin, 1% Rhozyme, and 1% pectinase ($\times 124$); **G** An individual Philippine Lacatan banana protoplast ($\times 1024$); **H** Palisade cells isolated from a mature Philippine Lacatan leaf by the enzymatic digestion of the middle lamella using 1% Rhozyme ($\times 103.5$); **I** An individual palisade parenchyma cell ($\times 1395$)

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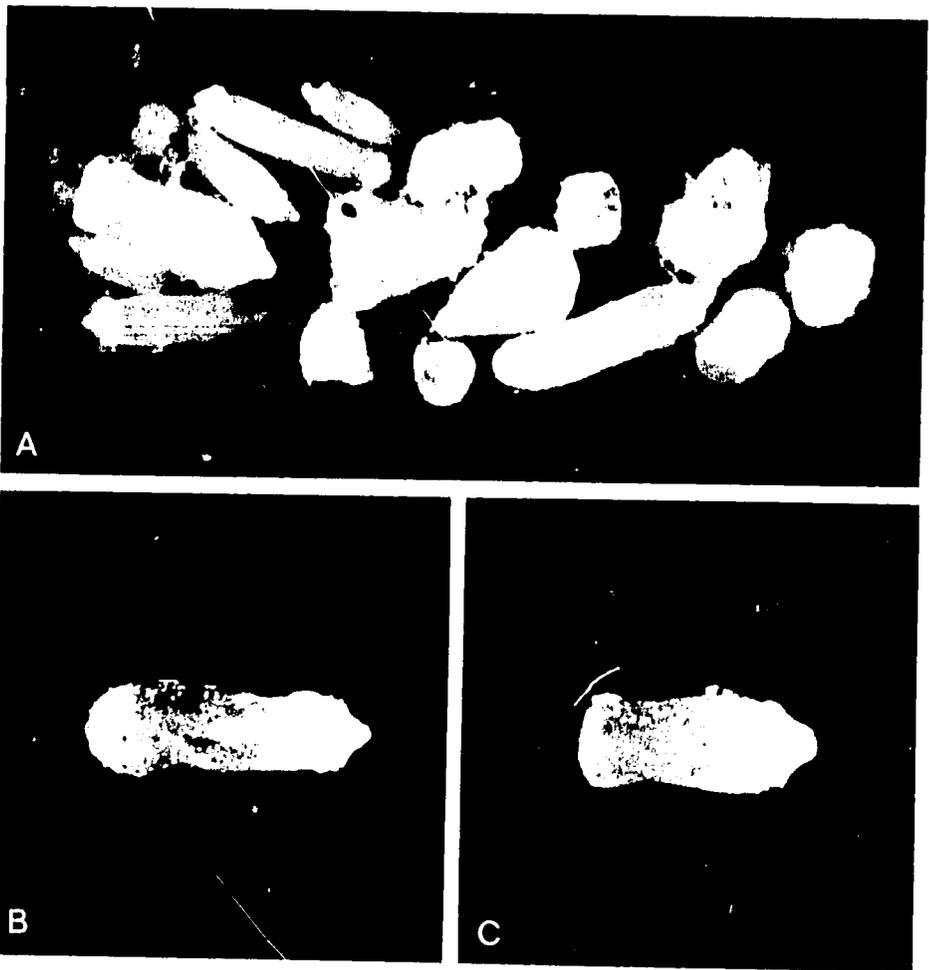


Fig. 3A - C. A Somatic embryos of *Cardaba* derived from cells which were, in turn, derived from multiplying shoot clusters cultured in B_5 + 1 mg l^{-1} 2,4,5-T and 1 mg l^{-1} BAP ($\times 5.8$); B and C Close-ups of somatic embryos of *Cardaba* ($\times 8.2$)

2.10 Regeneration and Transfer of Plants

Root formation can be readily induced on cultured *Musa* shoots. The individual shoots comprising a shoot cluster are usually separated first. Although Ma and Shii (1972) simply transferred shoots to sphagnum moss and allowed rooting to occur naturally, other investigators employed a number of auxins (see Table 1A) in order to assure that all of the shoots rooted. Berg and Bustamante (1974) found root formation to be slow process and while they tested IAA, NAA and IBA each at 1 mg l^{-1} , only NAA was effective. This supposedly was due to the presence of coconut water (10%) in their culture medium, the cytokinins naturally present in CW antagonized the root promoting properties of the auxins tested. It has been demonstrated that when coconut water is removed from the culture

medium, all three of these auxins are very effective at producing rooted plantlets from several clones (Cronauer and Krikorian 1984 b).

A number of investigators have added low levels of activated charcoal (AC) (0.1 to 1.0%) to their culture medium to counteract any deleterious effects which may result from the phenolic darkening of cut tissues. Subsequently, it has been observed that AC greatly stimulates root formation and this rooting procedure has been used by a number of investigators (Bower 1982, Hwang 1982 unpublished, Manicom 1982). An examination of the effects of AC has shown that when shoots are grown on a medium containing CW, the addition of increasing levels of AC increased the number of roots formed and overall root length. If AC is added to the medium which does not contain CW, the overall effect of increasing AC levels is to reduce the number of roots formed and increase root length (Cronauer and Krikorian 1984 b). We have found that the use of auxins such as NAA, IBA or IAA with extremely low levels of AC (0.025%) to be the most effective root inducing medium. When shoots are placed on such a medium, a sufficient number of roots are formed within 3 to 4 weeks to support the young plantlet when it is transferred to soil. Such plants are usually placed in a mist bed for 7 to 10 days before they are moved to normal greenhouse conditions.

2.11 Genetic Stability in Callus and Plants

Very little work has been done to evaluate genetic stability in callus or in plants. Chromosomes of *Musa* are small and difficult to work with (Vakili 1967). This, coupled with lack of morphogenetically competent callus, has presumably discouraged any serious effort to date. However, plants generated from shoot tip culture have been produced in large quantities by governmental, commercial and research laboratories. All accounts suggest that the materials generated are normal and do not differ in any detectable way from clones multiplied using conventional procedures. Whether more careful and more deliberate assessment would disclose any differences remains to be demonstrated. De Guzman and her colleagues sought to produce mutants with radiation, but none could be found (De Guzman et al. 1980). However, banana plants are known to occasionally give rise to sports or mutant suckers. In fact, some of the most important clones of commerce have arisen as spontaneous mutants. For example, Highgate is a dwarf mutant of Gros Michel (Simmonds 1966). Since relatively little has been done in this important area using mutagens or even variations in the conventional protocols of aseptic culture, one may still justifiably espouse the view that banana-plantain mutants can be produced in larger numbers than hitherto possible.

3 Conclusions and Prospects

Throughout this chapter the view has hopefully been reflected that aseptic culture techniques hold great promise for addressing the various problems related to banana and plantain improvement. The apical meristem, or more properly, the shoot tip culture procedures are playing a major role in facilitating rapid multiplication of desired clones of dessert banana. Far less effort has been placed thus far on cooking bananas and plantains, but this is sure to increase as world demands become more acute.

Several investigators have emphasized the benefits that could accrue if a means of producing dessert banana and plantain mutants could be worked out, applied and the products assessed (cf. Krikorian and Cronauer 1983). The little work that has been done thus far has involved γ -irradiation etc., prior to excision and culturing to the meristem or growing point (cf. Stotzky et al. 1964, Menendez 1973 a, b, Menendez and Loor 1979, Menendez and Shepherd 1975). Since the plantlets produced by aseptic meristem multiplication procedures are tiny, we see them as being very well suited to mutagenesis manipulation. If variants of the important Gros Michel or Grande Naine etc., could be produced, the task of export banana breeders might be made considerably easier. Similarly, the production of tetraploids by spontaneous endopolyploidization in diploid cultures or by use of colchicine (cf. Vakili 1962) could be useful. We know that multiplication via meristems, in itself and without mutagens, may have, under special conditions, potential for producing substantial variability in some crops. Whether this holds true for *Musa* is not known. Even if it does, one cannot, of course, be certain that variability will include resistance to such a disease as Black Sigatoka but resistance could be bred in.

The probability of selecting potential useful mutations via cell culture techniques would be greatly enhanced since many single cells could be affected and each of these would, theoretically at least, be capable of growing into a plant. Cell cultures can be grown in such a way as to produce variation – certainly vastly greater than that achievable by conventional breeding. If coupled with the use of mutagens, the possibilities of mutation breeding via cell suspension culture would be further increased. Protoplasts offer prospects of an even more sophisticated means of inducing or even introducing variation into a vegetatively propagated plant such as banana. Ideally, one would prepare protoplasts, expose them to mutagens and then rear as many of them as possible to plants. One could also envision, eventually, the possibility of protoplast fusion between edible diploids to make tetraploids. Cytoplasmic integration without concomitant nuclear integration would also be interesting, as would fusion of nuclei between unrelated clones, thus leading to production of somatic hybrids. Before one seriously anticipates even limited exploitation of any of these ideas, despite numerous facile claims for their usefulness in other crop plants, one must achieve a more complete understanding of all the aseptic culture methods as they relate to genus *Musa*. The attraction of the aseptic culture techniques, as they relate to bananas and plantains, is that methods already exist that can go far to meeting some of the more immediate needs. Simultaneously, the challenge of the requirement to lay the necessary groundwork for the much longer range and basic problems involving a true “genetic engineering” using full knowledge of the *Musa* genome must be met.

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REINITIATION OF VEGETATIVE GROWTH FROM ASEPTICALLY CULTURED TERMINAL FLORAL APEX OF BANANA¹

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ABSTRACT

Terminal floral apices of *Musa acuminata* cv. Dwarf Cavendish were isolated and cultured in a modified Murashige and Skoog (1962) medium supplemented with N⁶-benzylamino purine (5 mg/L) and 10% (v/v) coconut water. Under these conditions, the determinate floral buds are transformed into a multiplying vegetative shoot system. Rooted plantlets were obtained by treating shoots with the auxin, naphthaleneacetic acid (1 mg/L), and activated charcoal (0.025%). Practical benefits resulting from these observations, such as the production of virus-free plants and the rapid multiplication of stock material, are discussed.

WITH THE ONSET of flowering, the apical meristem of a banana plant ceases initiating foliar organs and begins to produce floral primordia. This transition from indeterminate to determinate growth marks the end of vegetative growth since flower and fruit formation becomes the last activity of an individual shoot meristem. This large monocotyledonous herb has a pseudostem comprised of layers of concentrically arranged sheathing leaf bases with radiating laminae that emerge from a perennial rhizome or corm (Simmonds, 1966). Mature banana plants consist of a clump of such pseudostems, but one shoot inevitably takes the lead thereby relegating the other shoots to the status of followers or suckers. In the vegetative condition, the underground corm or rhizome is the true stem and only in the development of the flowering shoot does the stem become conspicuous. At the "shooting stage," when the fruiting stalk emerges from the hollow of the pseudostem, the stem is terminated by a conspicuous purple knob of bract-covered sterile male flowers. In dessert bananas, the male knob is followed by a substantial expanse of naked and scarred stem and then a series of developing hands of fruit (the bunch) comprised of fingers (Alexandrowicz, 1955). In cultivation, when the bunch is cut, that individual shoot does not again bear fruit but provides nutrient to the suckers. One of these suckers will, in its turn, take the lead of growth.

It has long been recognized that flower for-

mation is critical in the life of the banana because very early on, the number of hands in the future bunch is irrevocably determined. Although the term *monocarpic* (Hildebrand, 1881) is not strictly applicable to bananas, since complete exhaustion of the entire plant does not occur, each individual shoot behaves like a monocarpic plant. Growth of the vegetative phase is limited or determined by the initiation of the floral phase of development (Barker and Steward, 1962b).

In this paper, we show that this seemingly determined state may be reversed and renewed vegetative growth may be initiated from the floral bud apex.

MATERIALS AND METHODS—'Dwarf Cavendish' banana plants, a dessert clone (AAA group according to the convention of Simmonds and Shepherd, 1956) were grown to fruiting size (2.0 m tall) in 54 cm × 50 cm pots under greenhouse conditions. After the fruit bunch had emerged, flower formation was allowed to proceed through the transition from female to male flowers. The terminal bud was cut from the peduncle (Fig. 1), and the bracts with their associated hands of male flowers were removed in a stepwise manner until they became too small to remove by hand (Fig. 2). Working with a dissecting microscope, scalpel, and forceps, the remaining bracts and minute hands of flowers were removed until the rounded growing point was revealed (Fig. 3). The apex and approximately 1 cm of subtending peduncle tissue were excised and surface sterilized by soaking in a 2% commercial bleach solution (final concentration 0.1% NaOCl) with two drops of Tween 20 for 5 min. The tissue was rinsed four times with sterile distilled water. Working under aseptic conditions, the apical dome, measuring approximately 0.5 mm in length, was

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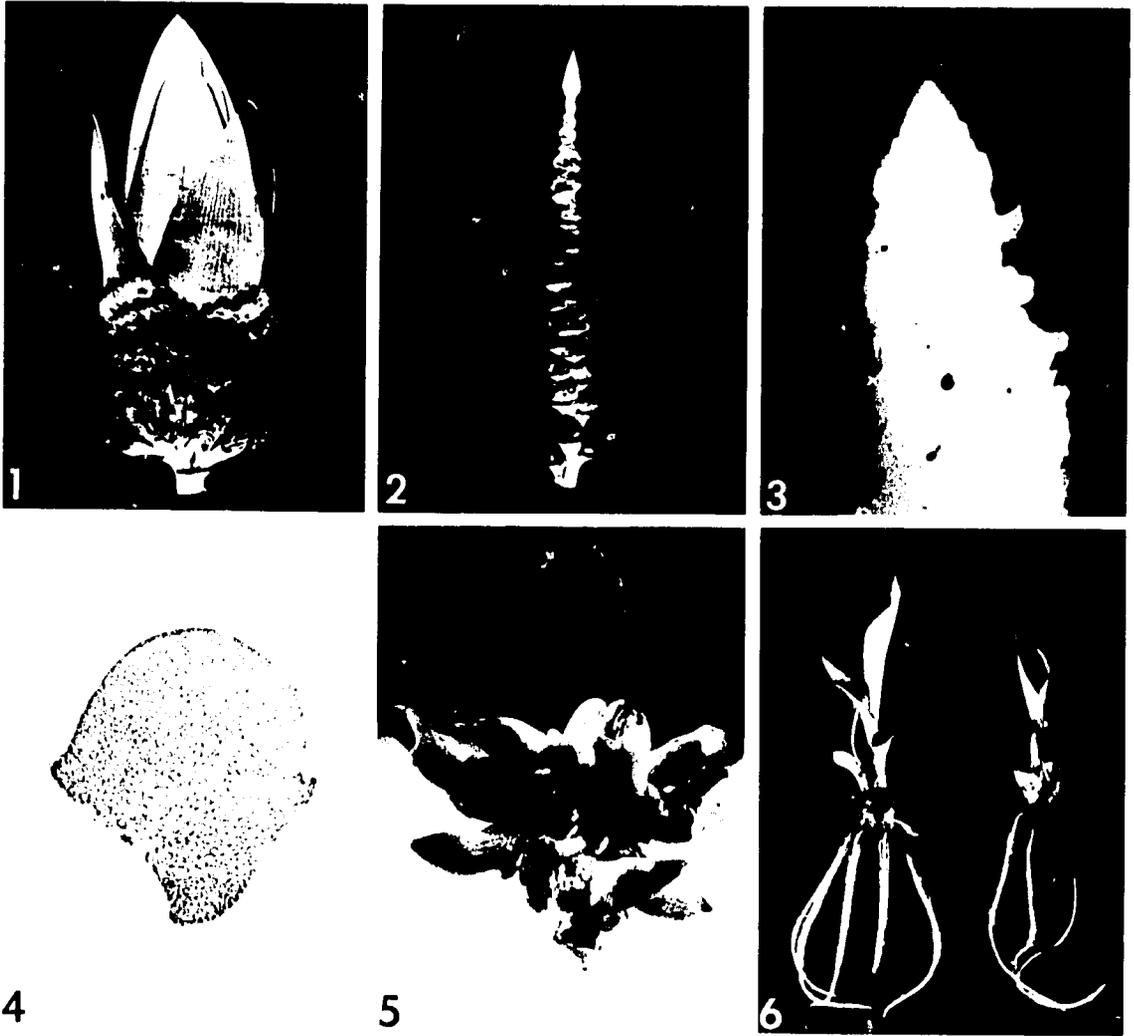


Fig. 1-6. Stages in the isolation and culture of a banana floral apex and subsequent production of rooted plantlets. 1. Terminal floral bud removed from the distal end of a 'Dwarf Cavendish' fruit bunch. $\times 0.15$. 2. The same bud with bracts and floral buds removed to reveal peduncle. The floral apex is still concealed under several layers of bracts and flower clusters. $\times 0.15$. 3. Exposed floral apex. $\times 6.5$. 4. Median longitudinal section of an isolated meristem from the floral apex. Note the absence of subtending flower and/or bract primordia. $\times 78$. 5. Shoot cluster derived from cultured floral apex 18 wk after isolation. $\times 1.2$. 6. Rooted plantlets produced by culturing individual shoots on medium containing 1 mg/L NAA for 21 days. $\times 0.45$.

removed by making two scalpel incisions into the subtending tissue (Fig. 4), and placed in liquid culture medium composed of the mineral salts of Murashige and Skoog (1962) and iron of Singh and Krikorian (1980), 100 mg/L inositol, 1 mg/L thiamine HCl, 5 mg/L N^6 -benzylaminopurine, 4% sucrose, and 10% (v/v) coconut water. The pH of the medium was adjusted to 5.8 with KOH prior to autoclaving at 121°C and 103 kPa for 20 min. Isolated apices were initially cultured in 20 ml of liquid medium in foam-stoppered 125-ml Erlenmeyer flasks and placed on rotary shakers at 80 rpm. This was alternated with semisolid me-

dium (0.65% Difco agar) of the same composition. All cultures were maintained in growth chambers at 30°C under a 16:8 L:D cycle (10.2×10^3 l m/m² Sylvania Grolox wide spectrum).

Multiplying shoot clusters were subdivided with a few scalpel incisions and maintained on semisolid medium in 6.5-cm \times 6.5-cm \times 10-cm plastic vessels (GA 7 vessels from Magenta Corp., Chicago, IL) and were routinely subcultured at 3-4 wk intervals. Roots were induced by transferring individual shoots to semisolid medium, which contained naphthaleneacetic acid (1 mg/L) as the sole growth

regulator with 0.025% activated charcoal. For ease of handling, this procedure was carried out using 18 mm × 150 mm Kaput tubes (Bellco Glass, Inc., Vineland, NJ), each of which contained 10 ml of medium.

Apices isolated for histological study were fixed in 3% glutaraldehyde, embedded in Paraplast, sectioned at 6 μm, and stained with safranin fast green.

RESULTS AND DISCUSSION—Apices grew slowly at first and were initially maintained in liquid medium for the first 2 mo with the medium being changed once. For the next four transfers, apices were alternated between semisolid and liquid medium at 2 wk intervals. This regimen promoted rapid growth and expansion of the newly formed shoot as well as multiple shoot formation from a single isolated apex. This development is similar to that reported for the culture of vegetative apices of a number of banana and plaintain clones (Cronauer and Krikorian, 1984). Four months after the initial explant was taken, the shoot cluster was large enough to be subdivided and maintained on semisolid medium (Fig. 5). Thereafter, multiplication proceeded at a rapid rate.

Overall shoot morphology of the multiplication stage was indistinguishable from that of material originating from vegetative apices placed into culture at the same time as well as vegetative apices of other triploid dessert banana clones (Cronauer and Krikorian, 1984). The apices became chlorophyllous and formed tiny pointed shoots as the leaf primordia developed. Within 2 mo, multiple shoot clusters arose in an identical manner as previously described for cultured vegetative apices (Cronauer and Krikorian, 1984). By subdividing the clusters and maintaining them on semisolid medium, a potentially unlimited number of shoots could be obtained. Rooting also proceeded in a manner similar to that of shoots arising from vegetative apices with the first roots appearing 5 days after shoots were placed on auxin-containing medium. Within 3 wk, the young plantlets were large enough to be transferred to the greenhouse (Fig. 6).

The morphogenetic plasticity of the growing point of a fruit bunch was first noted by Joshi (1939) who observed that if fully formed but immature fruits were removed from the peduncle, the apex, which had already been producing male flowers, could be induced to produce a few additional female flowers. The malleability of the banana apex as indicated by Joshi's work prompted us to wonder how the floral apex would respond if we exposed it

to a regimen designed for multiplying vegetative apices *in vitro*.

Although it should be noted that the true apical dome of the banana inflorescence is not strictly floral in the sense that it retains its identity while the bracts and flower clusters form along its flanks, the resultant production of vegetative shoots suggests that the pattern of cell division in the isolated apex underwent a change so that the quiescent center of the vegetative apex of banana with its flanking areas of leaf formation reappeared. Whether this may have derived from the direct application of cytokinins to the apex in the culture process or by the isolation of the extreme apical dome away from the stimuli of other closely associated cell layers and floral primordia has not been determined. *In situ*, the so-called "determined" state of a banana meristem may be exactly that, but the cells comprising the true apex are not irreversibly programmed at least to the extent that they are still capable of producing a vegetative shoot system when provided with the correct stimulus or environment. Examples of similar phenomena are to be found in *Impatiens* where floral buds have been induced to revert to a vegetative growth mode *in situ* (Krishnamoorthy and Nanda, 1968) and in *Selaginella*, where excised root meristems were redetermined as shoots *in vitro* (Wochok and Sussex, 1976).

Practical benefits arising from this procedure derive from both the location of the growing point and its morphology. Bananas and plaintains are subject to a number of infections, such as bunchy top and cucumber mosaic virus (CMV) (Stover, 1972; Wardlaw, 1972). The use of meristem culture in conjunction with thermotherapy has been reported to be effective in the eradication of CMV from banana plants (Berg and Bustamante, 1974). However, the isolation of a true apical meristem is hindered by the fact that the vegetative apex lies in a slight depression and is completely encircled by the youngest leaf primordia (Barker and Steward, 1962a). Because the floral apex appears as a dome of tissue atop a true stem with expanding internodes, it is elevated above the floral primordia making the isolation of the true meristem without any subtending primordia a relatively easy procedure. This could provide a means for more easily and reliably producing virus-free plants from the culture of a true meristem. Additionally, plants already having produced a fruit bunch that can be qualitatively judged in terms of taste, evenness of ripening, etc., can be placed into culture without sacrificing or disturbing any suckers of the

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mat. This could be especially useful to breeding programs where individual progeny of a cross are being assessed and an extremely limited amount of material is available for further testing or subsequent distribution (Rowe, 1984). With the disease Black Sigatoka, caused by the fungus *Mycosphaerella fijiensis* var. *difformis*, threatening the major producing areas of the world, especially Central and South America, renewed interest has been placed on the development of superior, more disease resistant or tolerant clones (Krikorian and Cronauer, 1984). A rapid multiplication method such as the one described here could readily produce additional planting material for field evaluation and subsequent release to growers.

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Aseptic Multiplication of Banana from Excised Floral Apices

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Additional index words. tissue culture, micropropagation, *Musa*

Most economically important bananas and plantains are large triploid seedless herbs that must be propagated vegetatively by removing small side shoots or "suckers" from the parent plant or by planting seed pieces of larger corms. Consequently, multiplication of stock material is time consuming. Recently, the rapid production of young banana plantlets suitable for use as "seed" material has been described (1, 3). Vegetative shoot apices were isolated and multiplied using aseptic tissue culture techniques. Although these multiplication systems, once established, can produce thousands of plants in a relatively short period of time, their establishment necessitates the initial sacrifice of an individual specimen, which may not always be desirable or prudent should a limited parent stock be available. We describe here the production and multiplication of rooted banana plantlets from the isolation and culture of terminal floral apices.

Plants of the dessert banana (*Musa acuminata* Colla) clone 'Dwarf Cavendish' [designated AAA by Simmonds and Shepherd (5)] were grown in the greenhouse to fruiting size in 54 × 50 cm pots. After the flower bud had emerged from the pseudostem, growth of the fruit bunch was allowed to proceed until all female hands of fruit had appeared. The large terminal floral bud then was removed, and the male flowers with their

subtending bracts were removed in a stepwise fashion. The final minute flower clusters and bracts were removed with forceps and scalpel under a dissecting microscope to reveal the elevated apical dome. The distal 0.2 mm of the floral apex was excised by making 2 angled cuts into the subtending tissue. It then was surface sterilized by soaking in 50 ml of a 0.105% aqueous solution of NaOCl (2% v/v Clorox) with 2 drops of Tween 20 for 5 min and rinsed 4 times with sterile distilled water. Disinfested apices were cultured in liquid medium composed of the mineral salts of Murashige and Skoog (4) supplemented with 5.55 mM inositol, 2.97 μM thiamine HCl, 0.12 M sucrose, 10% v/v coconut water, and 22.0 μM BA. The pH of the culture medium was adjusted to 5.8 with KOH, and 20 ml were poured into 125 ml Erlenmeyer flasks and autoclaved for 20 min at 127°C and 1.1 kg cm². Cultures were maintained on rotary shakers at 100 rpm in growth chambers at 30° in a 16 hr:8 hr L:D cycle at 10.2 Klux (Sylvania GroLux wide spectrum).

Isolated apices in a liquid medium grew slowly for 2 months into tiny green shoots. Shoot multiplication was stimulated by transferring tissue pieces alternately between liquid and semisolid medium of the same composition at 2 week intervals as previously described (1). The medium (50 ml in 100 ml screw cap jars) was solidified with 0.7% Difco Bacto agar. Thereafter, shoot cultures were maintained by subdividing multiplying shoot clusters with a few scalpel incisions and transferring them to fresh semisolid medium at 3 week intervals (1). Plantlets were rooted by placing individual shoots on semisolid medium containing 5.5 μM NAA and 0.025% activated charcoal. The first roots appeared within 5 days, and plantlets could be potted in Pro

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Mix:vermiculite (v/v) within 3 weeks. Potted plants were placed under intermittent mist for 1 week before transferring to normal greenhouse conditions.

Since bananas are subject to a number of viral diseases, such as bunchy top and cucumber mosaic (6, 7), the extreme ease with which floral apices of banana can be isolated, in comparison with vegetative apices, could facilitate the production of virus-free plantlets. The multiplication system described also could be a great help in a breeding program, since large numbers of a particular clone could be generated rapidly shortly after the fruit bunch had been evaluated. Further field trials then could be ac-

celerated while simultaneously leaving the parent plant and its slowly growing side suckers intact. This system could be particularly useful with those clones that are known to sucker at an extremely slow rate (2).

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Multiplication of *Musa* from Excised Stem Tips

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ABSTRACT

Rapidly multiplying cultures were established from excised shoot tips of two dessert banana clones ('Philippine Lacatan' and 'Grande Naine') and two plantain clones ('Pelipita' and 'Saba'). Apices cultured on semi-solid media produced single shoots while apices placed in liquid media produced shoot clusters. Individual shoots were induced to form multiple shoot clusters by longitudinally splitting the shoot through the apex. Shoot multiplication was stimulated maximally by 5 mg l⁻¹ benzylaminopurine. Rooted plants were produced by treating shoots with auxins. Growth rates based on increase in f. wt of the four clones were compared. During a 4-week culture period 'Pelipita' showed a fivefold increase in f. wt while 'Grande Naine', 'Philippine Lacatan' and 'Saba' showed increases exceeding tenfold.

Key words: *Musa* cultivars, bananas, plantains, tissue culture, clonal multiplication.

INTRODUCTION

Clones of *Musa* which yield edible fruit grow from massive underground corms with highly compressed internodes. The growing point is located at the centre of the crown of the corm. Growing upward from around the apex are the leaf sheaths which encircle each other and comprise the pseudostem which, in turn, supports the laminae. When flowering occurs, the internodes lengthen and the true stem is pushed up through the pseudostem to form a long peduncle (Barker and Steward, 1962).

Since virtually all of the edible clones are parthenocarpic and for all practical purposes seedless or seed sterile, multiplication must be vegetative. Materials which can be used for propagation include 'button buds' which may arise at the sides and around the crown of the corm, young shoots called 'sword suckers', which arise at the base of the main 'trunk', and portions of the corm, so-called 'bits'. Sword suckers are the preferred type of propagule since they produce fruit more quickly than smaller suckers or pieces of the corm (Eastwood, 1930; Simmonds, 1966 p. 161).

Despite the availability of these various propagules, there has often been a shortage of planting material which rigorous quarantine regulations have further exacerbated (Stover, 1977). Consequently, various systems for the maximum multiplication of planting material have been developed. Barker (1959) examined the potential for increased suckering by stripping the older leaf sheaths from the pseudostem, thus permitting buds that do not normally grow under field conditions to develop. Several other means of stimulating bud development have also been reported (cf. Hamilton, 1965; Ascenso, 1967; Martinez, 1978) but the stripping method of Barker remains the one of choice.

There is little detailed literature on the multiplication of various *Musa* clones by means of aseptic culture techniques. Some of these deal with the production of specific pathogen-free plantlets by thermotherapy (e.g. Berg and Bustamante, 1974 for eliminating cucumber mosaic virus); others deal with multiplication and micropropagation (Ma and Shii, 1972; deGuzman, 1975; deGuzman, Ubalde and Del Rosario, 1976; Bower, 1982;

Dore Swamy, Srinivasa Rao and Chacko, 1983). In this paper, we discuss the establishment and characterization of multiple shoot cultures and the production of rooted plantlets. The method can yield thousands of young rooted plantlets in a short period of time. We have selected four important *Musa* clones, two dessert bananas ('Philippine Lacatan' and 'Grande Naine') and two plantains ('Pelipita' and 'Saba'), for study. 'Pelipita' and 'Saba' are particularly interesting since they both possess a high tolerance to Black Sigatoka disease caused by the fungus *Mycosphaerella fijiensis* var. *difformis* (Stover, 1980; Rowe, 1984) which is currently threatening worldwide banana and plantain production. Consequently, there is an increased demand for planting material of clones such as 'Saba' and 'Pelipita' and a rapid multiplication technique such as is described here would easily satisfy that need.

MATERIALS AND METHODS

Initiation and maintenance of cultures

Cultures were established by isolating shoot apices from greenhouse-grown plants 1.5 m tall. 'Philippine Lacatan' is from the Philippines; the others are of Honduran origin. Successive sheathing leaf bases were removed by hand (Fig. 1A) and with the aid of a dissecting microscope the apical area with one or two leaf primordia (approx. 1–2 mm in length) was removed together with a piece of subtending corm tissue. The apical tissue was sterilized by soaking for 5 min in 50 ml of a 1 per cent Clorox solution (0.05 per cent NaOCl) containing two drops of Tween 20, swirling occasionally. The shoot tip was then rinsed four times with sterile distilled water. The culture medium contained the mineral salts of Murashige and Skoog (1962), with iron chelate (Singh and Krikorian, 1980), 1 mg l^{-1} thiamine HCl, 100 mg l^{-1} inositol, and 4 per cent sucrose, hereafter abbreviated B_{MS} . The medium was supplemented with 5 mg l^{-1} benzylaminopurine (BAP) at pH 5.8. Autoclaving was carried out at 121°C and 103 kPa for 20 min. This medium was used either as a liquid or solidified with 0.7 per cent Difco agar. Shoot tips which were first cultured in liquid medium on a gyratory shaker at 100 rev/min were transferred to semi-solid medium after 21–30 d. Established cultures were routinely transferred to fresh semi-solid medium every 3–4 weeks by subdividing shoot clusters with a scalpel. All cultures were maintained in a growth chamber at $30 \pm 1^\circ\text{C}$, 50 per cent r.h. on a 16-h light cycle ($10.2 \times 10^3 \text{ lm m}^{-2}$ Sylvania GroLux wide spectrum).

Comparison of liquid and semi-solid culture medium

Shoot tips of several clones were grown in both liquid and semi-solid culture media for 20 d, then those shoots in liquid were transferred to a semi-solid medium. Following an additional 22 d, shoots were weighed and the number of individual shoots comprising a cluster counted.

Shoot multiplication

Shoot multiplication was induced by cutting a single shoot (2 cm tall or larger) longitudinally through the apex. The effect of the cytokinins BAP and KN added alone and in combinations of varying levels, were tested on clone 'Pelipita' (Table 1). *Tupidanthus* medium (Murashige, 1980) which contains 3 mg l^{-1} BAP and 0.3 mg l^{-1} IAA was also tested. One split shoot (two shoot halves) was placed upright in a 100 ml screw cap jar containing 50 ml of semi-solid medium. After 4 weeks, the shoot number, shoot length, root number and root length were determined and averages were computed. Each treatment had five replicates of two shoot halves.

TABLE 1. *Multiplication of Musa clones from established shoot cultures over a 28-d period (f. wt increase (g), average of 16 replicates)*

	Initial wt	Days in culture			
		7	14	21	28
'Saba'	0.73 ± 0.22	1.45 ± 0.43	3.02 ± 0.98	4.98 ± 1.72	7.98 ± 3.02
'Grande Naine'	0.46 ± 0.13	1.06 ± 0.37	1.96 ± 0.84	3.46 ± 1.56	5.72 ± 2.71
'Pelipita'	0.50 ± 0.21	0.80 ± 0.30	1.22 ± 0.41	1.87 ± 0.69	2.93 ± 1.18
'Philippine Lacatan'	0.24 ± 0.06	0.55 ± 0.13	0.99 ± 0.39	1.59 ± 0.75	2.62 ± 1.44

Determination of growth rates

The growth rates of established branching shoot cultures of four clones ('Pelipita', 'Saba', 'Philippine Lacatan' and 'Grande Naine') were determined using the normal maintenance procedure. Small clusters of shoots representative of the tissue being subcultured were weighed aseptically at weekly intervals and returned to their culture vessels. Sixteen replicates were used for each clone.

Root induction

The root inducing effects of the auxins NAA, IBA and IAA each at 1 mg l⁻¹ was examined with and without coconut water (10 per cent v/v, CW) and two levels of activated charcoal (0.025 and 0.25 per cent, AC). Two shoots were placed in each 100 ml screw cap jar containing 30 ml of medium. Each treatment had six replicates. After 4 weeks, observations were made.

RESULTS

Establishment of cultures

Isolated shoot tips, creamy white when placed in liquid medium became green within 10 d and in 21 d, a small shoot was visible to the naked eye. Shoot tips on semi-solid medium responded in a similar manner but at a slower rate. Those cultured in liquid for a total of 3–4 weeks grew to 1–1.5 cm long and were then transferred to semi-solid medium of the same composition. These shoots went on to produce clusters of multiple shoots which could be subcultured. Shoot tips initially cultured on agar yielded single shoots, rarely multiple shoots (Fig. 1B). In one experiment, shoot tips initially placed in liquid medium produced an average of 6.6 shoots per cluster weighing an average of 2.5 g while the corresponding shoot tips placed on semi-solid medium each grew into a tiny single shoot with an average weight of 20 mg.

Shoot multiplication

Four to 7 d after a shoot was split to induce multiplication, the outer darkened leaves and shoot base were trimmed away to reveal new side shoots (Fig. 1C) and the halves were transferred to fresh medium. After an additional 2–3 weeks, the new multiple shoots were separated with a scalpel and transferred again. From this point on, transfer was needed at 3-week intervals since the proliferating cultures filled the vessels.

The level of cytokinin added to the medium greatly affected the number of new shoots



formed (Fig. 2). When a split shoot was placed on B_{MS} with no hormones, about two rooted shoots formed from each half and further shoot multiplication was inhibited. The number of shoots formed could be increased without concomitant root formation by the addition of 5 mg l^{-1} BAP to an average of 9.1 new shoots per shoot half. Kinetin also increased the number of new shoots formed but it was not as effective in inhibiting root formation. When both cytokinins were used, the BAP/KN ratio was not as important as maintaining the total level of exogenous cytokinin at 5 mg l^{-1} . Increasing the cytokinin level beyond 5 mg l^{-1} failed to produce more shoots. *Tupidanthus* medium, containing 3 mg l^{-1} BAP and 0.3 mg l^{-1} IAA, used at a commercial nursery to produce young banana plantlets, was not as effective as BAP at 5 mg l^{-1} or the BAP-KN combinations totalling 5 mg l^{-1} . In treatments which produced rapidly multiplying shoot clusters and no roots (i.e. B_{MS} plus 5 mg l^{-1} BAP, B_{MS} plus 5 mg l^{-1} each of BAP and KN, B_{MS} plus 3.75 mg l^{-1} BAP and 1.25 mg l^{-1} KN, and *Tupidanthus* medium), the average shoot length did not exceed 1.9 cm. In treatments where KN was used alone or the BAP/KN ratio was low (i.e. B_{MS} plus 1.25 mg l^{-1} BAP and 3.75 mg l^{-1} KN), roots formed and the average shoot length increased. In the control treatment (B_{MS}), long roots formed and shoot height reached its maximum (Fig. 2).

Growth rates

Throughout the 4-week subculture interval, all four clones demonstrated large increases in f. wt (Table 1). After a 4-week culture period, the clone 'Pelipita' showed

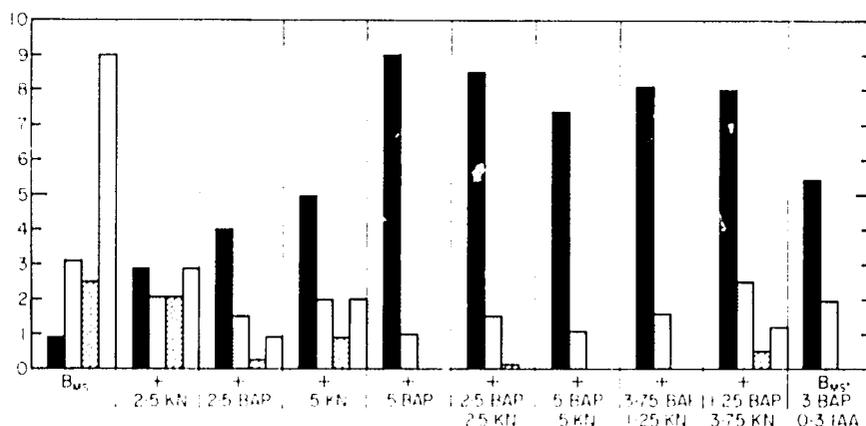


FIG. 2. The effect of cytokinins on the number and length (cm) of shoots and roots of 'Pelipita' following 4 weeks in culture: ■, shoot number; □, shoot length; □, root number; □, root length. The initial inoculum comprised one-half of a young shoot obtained by longitudinally splitting an intact (but unrooted) shoot about 3 cm tall. Data represent ten replicate shoot halves. Note: in B_{MS} plus 2.5 BAP and 5 KN, one of the ten shoot halves produced a 1.3 cm long root. B_{MS} supplemented with 85 mg l^{-1} monobasic sodium phosphate and 40 mg l^{-1} adenine sulphate.

FIG. 1. Stages in the development of multiple shoot cultures of and root formation in various *Musa* clones. A, exposed growing point of 'Philippine Lacatan' prior to excision. $\times 2.7$. B, isolated growing points of 'Philippine Lacatan'. Left, cultured for 42 d on semi-solid medium; right, cultured in liquid for 20 d followed by an additional 22 d on semi-solid medium. $\times 1.3$. C, shoot of 'Philippine Lacatan' split longitudinally through the apex to induce formation of new side shoots. $\times 1.0$. D, a large shoot cluster of 'Pelipita'. $\times 1.1$. E and F, the effect of CW and AC on root initiation and growth of 'Pelipita' shoots. In each photograph, the plantlets are arranged left to right, 0, 0.025 and 0.25 per cent AC. Note that the inhibition of rooting caused by BAP and CW was readily reversed by AC whereas AC had no effect on the auxin treatment. $\times 0.3$. E, B_{MS} plus 1 mg l^{-1} IBA; F, B_{MS} plus 1 mg l^{-1} IBA and 10 per cent CW. For abbreviations see text.

a fivefold increase in f. wt while 'Grande Naine', 'Philippine Lacatan' and 'Saba' all showed increases in f. wt exceeding tenfold.

Rooting

Root formation could be induced in a number of ways, forming in as few as 4 d. However, the responses differed markedly in the number of roots formed and their length. Individual shoots were induced to root by eliminating all hormones from the basal medium. Adding AC increased the average root length, but not root number. When CW or BAP was added to the culture medium, root formation was inhibited. This inhibition was reversed by the addition of small amounts of AC (0.025 per cent).

Each of the three auxins, IAA, IBA and NAA promoted abundant root formation. Activated charcoal showed little effect on the root promoting abilities of these treatments (Fig. 1 E). The combination of CW with the auxin greatly reduced root number and length. Once again AC readily overcame this inhibition as both root number and length increased (Fig. 1 F). Rooted shoots have been potted in a soil:vermiculite (1:1) mixture in small plastic pots, placed in a misting bed for 7–10 d, and then transferred to normal greenhouse conditions. Plants multiplied by the procedures outlined here have routinely been grown to 1.5 m in height in the Stony Brook greenhouses. Survival from culture vessel to soil has been 100 per cent. The performance in the field of plants produced using these techniques is under evaluation.

DISCUSSION

Berg and Bustamante (1974) reported the isolation and culture of banana meristems on Knudson's medium supplemented with casamino acids (1 g l^{-1}) and 10 per cent (v/v) CW. In our early studies on stem tip culture of *Musa* clones, we found that when shoot tips were cultured on our B_{MS} medium supplemented with BAP, complex additives such as these were unnecessary. In Berg and Bustamante's experiments, each meristem produced one shoot. When isolated shoot tips were placed on semi-solid medium, we obtained the same results. Dore Swamy *et al.* (1983), working with much larger apical explants also obtained single plantlets. We have shown that multiple shoot cultures can be produced on agar medium by splitting a small shoot longitudinally through the apex or by culturing apices in liquid medium. Previously, the only reported advantage of liquid media (Ma and Shii, 1974) was that adventitious buds of banana which had remained dormant on solid medium for up to 10 months grew when transferred to liquid medium. Dore Swamy *et al.* (1983) approached the problem of producing multiple shoot cultures on agar simply by culturing much larger explants and thereby sacrificed the advantages conferred by working with true apical explants. Vessey and Rivera (1981) have produced branching shoot cultures by making several cuts into an isolated apical tissue piece. We have extended these studies by working with very small explants, getting much higher rates of multiplication and improving methods of root induction.

Rapidly proliferating shoot cultures were readily established on a number of media with one (B_{MS} plus BAP 5 mg l^{-1}) giving the best results. In 'Pelipita', for instance, one split shoot gave rise, on average, to 18 new shoots but we have seen a single split shoot yield up to 27 new shoots in 4 weeks. These cultures were easily subcultured by subdividing shoot clusters with a few scalpel incisions. Cultures have maintained their rapid rate of growth for over 4 years. In order to maintain this rapid proliferation rate, the shoot clusters have had to be subdivided, which in turn, fosters additional shoot growth. Thus, the potential to produce vast numbers of shoot material very rapidly can be realized.

Root production has been the slowest aspect of banana plantlet formation via aseptic

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cultures. Vessey and Rivera (1981) reported root formation occurred 50 d after shoot development. When Berg and Bustamante (1974) placed meristems on culture medium supplemented with NAA (1 mg l^{-1}), roots formed after 2–3 months on shoots about 1 cm tall but these shoots did not elongate to 4–5 cm in height until they were 3–4 months old. We have found that NAA, IAA and IBA are all equally effective in inducing root formation and the addition of CW to the rooting medium reduces or antagonizes the auxin's activity thereby explaining Berg and Bustamante's slow rooting response. Presumably, this is due to the cytokinins naturally present in CW (Steward and Krikorian, 1971 p. 88). Omitting CW from the culture medium or the addition of small amounts of AC (approx. 0.025 per cent) greatly enhanced root formation.

We have observed that when roots are induced on shoots early in the culture process, there is less tendency to produce additional shoots. Instead, the young plantlet will increase in height and the leaf blades will begin to expand. To maximize the number of shoots generated from a single shoot tip (e.g. if a new clone were being propagated), the formation of roots should be discouraged until the individual shoots have been separated. Through repeated subculturing of proliferating shoots, an open ended system can be maintained. Although transfer to a separate rooting medium may require additional labour, roots will form rapidly. Even though measurements on our rooting experiments were carried out at 4 weeks, sufficient number of roots formed within 2–3 weeks to support continued growth of young plants when potted in soil and placed in mist. Therefore, total time from the isolation of a shoot tip to the establishment of rooted plantlets can be as short as 7 weeks. If an open ended system is sought, the accumulation of additional shoot material will necessarily take longer depending on the scope of the operation desired. We do believe, however, that aseptic shoot multiplication may be more successful with some clones than with others since we have encountered variability among other clones with respect to the degree of shoot multiplication induced. In the case of the two plantain clones discussed in this paper, 'Pelipita' produced fewer new shoots than did 'Saba' and this was reflected in this increase in f. wt over a 4-week period. We also believe that the source of the initial inoculum (for instance, a shoot tip from a field-grown plant, a shoot tip from an aseptically cultured plantlet or an embryo from diploid seed) may affect the speed with which a rapidly multiplying, open-ended system can be established. Apices isolated from aseptically cultured shoots respond the quickest, while apices isolated from field-grown plants respond somewhat slower. This variation in response may indicate that the production of cytokinins in the roots helps to overcome the powerful apical dominance of the banana shoot tip and that the addition of exogenous cytokinins such as BAP is not always sufficient in establishing a multiplying shoot culture. Nevertheless, we still wish to emphasize that once cultures have been established, a potentially unlimited number of plantlets can be produced.

ACKNOWLEDGEMENTS

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Rapid Multiplication of Bananas and Plantains by *In Vitro* Shoot Tip Culture

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Additional index words. tissue culture, micropropagation, *Musa*

Abstract. Rapidly multiplying cultures of dessert banana clones ('Philippine Lacatan' and 'Grande Naine') and plantain clones ('Saba' and 'Pelipita') were established from isolated shoot tips on a modified Murashige and Skoog medium supplemented with 5.0 mg/liter 6-benzylamino purine (BA). The growth rates of these cultures, expressed as increase in fresh weight over a 4-week period, were assessed. Rooted plantlets were produced using the auxins naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), or indole-3-acetic acid (IAA) at 1 mg/liter with low levels of activated charcoal (0.025% w/v).

Bananas and plantains normally are propagated vegetatively using suckers of various sizes or pieces of the corm since the fruits of the edible clones are seedless. Since forcing buds under field conditions is a tedious and time-consuming procedure, the amassing of a sufficient amount of suitable planting material is slow at best (1, 8). This is a problem when a new clone is being generated to replace older plantings or when a large amount of planting material of a specific pathogen-tolerant clone is needed for field testing and assessment. The prevalence of disease problems and the need for generating clean planting stock in large quantities has stimulated recently a surge of interest in the production of clonal material of both cooking and dessert bananas by the use of aseptic micropropagation techniques (4). Published procedures emphasize the use of large shoot tip explants which are relatively slow to multiply (2, 5, 12). Moreover, the majority of effort has been placed on clones of dessert bananas destined for export (4).

We describe here a shoot multiplication procedure utilizing small primary explants which can produce thousands of clonal plantlets in a short period of time.

Apices from 2 important dessert banana clones ['Grande Naine' and 'Philippine Lacatan', AAA designation of Simmonds and Shepherd (9)] and 2 ABB plantains ['Saba' and 'Pelipita', ABB designation of Simmonds and Shepherd (9)] were isolated by removing the sheathing leaf bases which make up the pseudostem until the leaf sheaths be-

came too small to remove by hand. The remaining leaves were removed with the aid of a dissecting microscope, forceps, and a scalpel. Shoot tips (the vegetative meristem plus 1 or 2 leaf primordia) were removed and surface-sterilized for 5 min in a 0.0525% solution of NaOCl (1% commercial Clorox) with a few drops of Tween 20. They were rinsed then 4 times with sterile distilled water and transferred to culture medium comprised of the mineral salts of Murashige and Skoog (6), here abbreviated as B_{MS} , supplemented with (per liter) 100 mg inositol, 40 g sucrose, 1 mg thiamine · HCl, and 5 mg BA; pH was adjusted to 5.8 and the medium solidified with 0.7% Difco Bacto agar. Fifteen ml of medium were poured into 30-ml. French square, screw-cap glass jars which were autoclaved for 20 min at 127°C. Cultures were maintained at $30^{\circ} \pm 2^{\circ}$ at 50% relative humidity in a 16-hr daily illumination cycle at 10.2 Klx (Sylvania Gro-lux wide spectrum). Once the cultures had become established, they were moved into 100-ml screw-cap jars with 50 ml of medium and were subcultured routinely every 3 to 4 weeks by subdividing the shoot clusters with a few scalpel incisions and transferring the divided clusters to fresh medium.

The growth rate of established shoot cultures was monitored by weighing under aseptic conditions small shoot clusters representative of the type of tissue used during normal subculturing and placing them on maintenance medium. The tissue pieces were removed from the jars, reweighed, and returned to the same jar at weekly intervals. The increase in fresh weight and the increase as a percentage of original weight were calculated. Each clone had 16 replicates.

Isolated apices from each of the 4 clones almost always grew into individual small shoots (Fig. 1a). This was true even if they were cultured on media that contained high levels of cytokinins such as BA and/or kinetin. Multiple shoot cultures could be in-

duced by longitudinally splitting a young, cultured shoot through the apex and placing each half upright on semi-solid medium. Tests have shown that the addition of 5 mg/liter BA promotes rapid shoot multiplication (Fig. 1b) with each half shoot yielding, in the case of 'Pelipita', an average of 9.1 new shoots in 3 weeks. The other clones showed comparable rates of shoot multiplication.

Established cultures continue to multiply at a rapid rate. This is reflected by the increases in fresh weight during a 4-week culture period (Fig. 2). The 2 dessert banana clones ('Philippine Lacatan' and 'Grande Naine') and the plantain 'Saba' all had fresh weight increases in excess of 1000%, while the plantain 'Pelipita' showed a fresh weight increase of 500%. Although shoot cultures of 'Pelipita' did not multiply as rapidly as the other clones, it still yielded a very satisfactory number of new shoots.

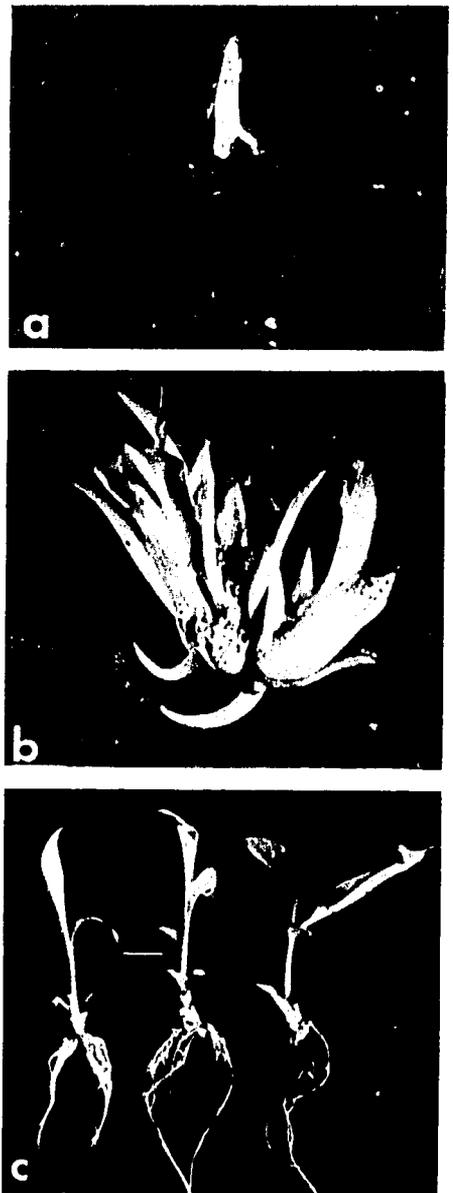


Fig. 1. Stages of shoot multiplication and plantlet production. a. 'Philippine Lacatan' banana shoot tip cultured for 34 days on semi-solid medium composed of B_{MS} + 5 mg/liter BA ($\times 2$); b. Representative piece of an established multiple shoot culture of 'Grande Naine' ($\times 1$); and c. Rooted 'Saba' plantlets 4 weeks after transfer to root-inducing medium ($\times 0.3$).

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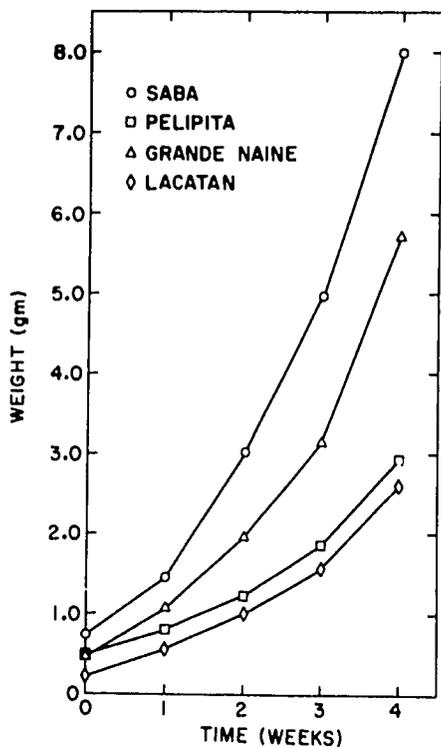


Fig. 2. Growth of shoot cultures of 4 *Musa* clones as reflected by the increase in fresh weight over a 4-week period. Each data point represents 16 replicates.

Plantlets were produced from all 4 clones by separating the clusters into individual shoots and transferring them to a root-inducing medium which contained, in addition to the basal medium, either NAA, IAA, or IBA at 1 mg/

liter with a low level of activated charcoal (0.025%). Roots generally appeared within 5 to 7 days. A sufficient number of roots had formed on 100% of the shoots after an additional 1 to 2 weeks so as to enable transferral to the greenhouse. All of the plantlets survived transferral to the greenhouse when rooting was allowed to proceed for a total of 4 weeks (Fig 1c). These plantlets were potted in 1 Pro Mix BX (Premier Brands, Inc., New Rochelle, N.Y.): 1 vermiculite in "3 square" plastic pots and placed in a misting bed on a 15 min cycle for 7 to 10 days. Afterwards they were moved to 23° day: 18°C night greenhouse conditions.

The major banana and plantain growing regions of this hemisphere currently are threatened by the spread of the disease 'Black Sigatoka' caused by the fungus *Mycosphaerella fijiensis* var. *difformis* (11). The clones 'Pelipita' and 'Saba' possess a high degree of resistance to this disease (11). There has been a widespread shortage of suitable planting material because of the increased demand for these clones. Strict quarantine regulations in producer-exporter countries further exacerbates the problems of widespread germplasm transfer (10). Implementation of a multiplication system such as the one described here could help alleviate these shortages. It also will be useful in the rapid distribution of any new disease-resistant clones which may be produced via conventional breeding or aseptic culture-based efforts (3).

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Aseptic Culture Techniques for Banana and Plantain Improvement^{1,2}

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There is an urgent need to identify or to produce 'Black Sigatoka' disease-tolerant or -resistant cooking and dessert bananas. Since bananas are perhaps the most conspicuously sterile of all cultivated fruits, breeding of resistant stock is fraught with great difficulties. An overview is provided of the potential value that may be derived from the use of aseptic culture techniques for generating and/or multiplying specific pathogen-tolerant clones. Special emphasis is given to the principles underlying various strategies and to the several levels of sophisticated methods presently available or that still need to be further developed before substantive practical benefits accrue. While the stance adopted in this paper is conservative, so-called tissue culture approaches to banana breeding and improvement may well serve as a model not only for Musa but for other recalcitrant crop plants.

Aseptic culture and modern molecular biological techniques are increasingly being seen as a means whereby crop and other economically important plants may be improved. The improvement ranges from such things as increase by clonal multiplication in the overall numbers of a given highly desirable or elite genotype/phenotype (Conger, 1981; Rao, 1982; Bonga and Durzan, 1982; Sen and Giles, 1983) all the way to laboratory genetically engineered plants which would purport to have important traits or qualities best considered as novel to say the least (Vasil et al., 1982; Kosuge et al., 1983). Some of the methodology is already in use, much of it is in an experimental research mode, and most of it in terms of integrated and coordinated usage is, at present, little more than optimistic expectation or even pious hope.

Conventional plant breeding and selection is, of course, the common vehicle whereby new cultigens are more or less routinely produced (Ferwerda and Wit, 1969; Simmonds, 1979). While this is acceptable strategy for those plants that are fertile and produce viable embryos, species that do not do so pose special problems. Mutation breeding in these instances has been the means for improvement. This has, like everything else, its own special set of challenges (Broertjes and van Harten, 1978; Gottschalk and Wolff, 1983).

The decision to explore the potential for use of such novel approaches in a given situation should not be taken lightly, of course, since the techniques are demanding, not inexpensive and must usually be developed de novo. Even so, there are few instances that we can think of where the "new technology," if it were available even for experimental application, might better play a role in crop improvement than in the case of bananas and plantains (Krikorian and Cronauer,

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1980

1984a,b).⁴ Edible varieties of *Musa* are among the most sterile of all cultivated plants. All have been selected from naturally-occurring, essentially seedless populations. In fact, no clone bred by man has ever been used on a commercial scale (Rowe, 1981, 1984).

Peasant farmers throughout the tropics and subtropics have apparently learned through experience that it is usually best to grow a number of clones to ensure availability of fruits for their everyday use, be it for cooking or consumption as fresh fruit. If a pathogen or pest problem emerges, there is usually some recourse from total disaster because of the variable germ plasm base. Commercially exploited plantings of dessert bananas (and plantains for that matter) are essentially monoclonal, however, and are thus highly and uniformly vulnerable to pathogens. This susceptibility was dramatically brought home, so to speak, by the important export dessert banana clone 'Gros Michel.' By the late 1950s 'Gros Michel' plantings were virtually wiped out or abandoned as a result of the Panama disease caused by *Fusarium oxysporum* f. *cubense* (Stover, 1972; Wardlaw, 1972). Availability of the Cavendish clones of dessert banana that are resistant to Panama wilt permitted replacement of the susceptible plantings, but once again the commercial operations are virtually monoclonal.

Now a different problem called 'Black Sigatoka' threatens much of the world's banana and plantain production. It is a leaf spot disease caused by the fungus *Mycosphaerella fijiensis* var. *difformis* and is raising havoc in many banana- and plantain-growing countries (Stover, 1980). While chemical spray programs can go far towards controlling the disease, they are so expensive that even large producers cannot bear the costs. 'Black Sigatoka' alone is costing the banana export industry more than \$100 mill/yr (R. H. Stover, pers. comm.).

To tackle the new objective of producing 'Black Sigatoka' (or any other disease for that matter) -tolerant or -resistant clones by conventional breeding based on hybridization is, at best, a long term project because it means that resistance present in wild diploid sources will have to be transferred into a commercially viable tetraploid. For those who do not have access to "advanced diploids," resistance may need to be found at the *beginning* of the long breeding sequence designed to end with a commercial polyploid (Pearson et al., 1983). Crossing with pollen from male fertile clones and flowers of male sterile, but female fertile, triploid clones has been the prime, but by no means only, strategy of banana breeders (DeLanghe, 1969; Rowe and Richardson, 1975). Tetraploid progeny, resulting from fusion of the triploid egg cell of the female and the haploid gamete

⁴ Hybridization between the wild, diploid ($2n = 22$) species *Musa acuminata* and *M. balbisiana* throughout their evolution has resulted in the emergence of cultivars of ploidy levels designated by various genome formulae. Using some 15 characteristics, Simmonds and Shepherd (1955) categorized many of the world's cultivars in terms of the genomic contributions made by each of the "parental" species. *M. acuminata* = genome 'A'; *M. balbisiana* = genome 'B.' Other naturally-occurring genotypes are 'AB,' 'AAA,' 'AAB,' 'ABB,' 'ABBB' and 'AAAA.' The predominant clones of commerce are triploid and sterile, producing fleshy, seedless fruits through parthenocarpy (Simmonds, 1962, 1966, 1976). The designation *plantain* has been widely applied to the 'AAB' and 'ABB' clones of starchy bananas that are eaten only when cooked, i.e., not as a dessert fruit. There is now some effort to distinguish between these 2 cooking groups by referring to only the 'AAB' clones as plantains and to the 'ABB' clones as cooking bananas. We have not felt the need to draw such a fine distinction in this article.

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of the male parent, are produced in small numbers because of the miniscule number of seeds produced (Rowe, 1981, 1984). About 3 yr is required before one sees the results of a cross and some have even estimated that 10 yr from initial development might be needed before a suitable 'Black Sigatoka'-tolerant export banana could be bred. In addition to the fact that there can be no guarantee that such breeding would necessarily lead to a marketable end product, there is the additional problem that conventional breeding cannot possibly keep pace with the development of any new, and potentially more virulent strains of pathogens. The approach of attempting to introduce new edible clones that have hitherto been popular only in localized areas and that have desirable features such as resistance, etc., is optimistic and has its own special problems including consumer acceptance or marketability. The latter is an important consideration, for export bananas grown for the Western market pose special problems. They must conform to rather stringent requirements such as shape, taste, ripening pattern, etc., and it is unrealistic to think that a resistant dessert clone(s) that combines many exceptional features will be found quickly. (The chances of identifying acceptable cooking banana clones by this means is much greater in our view.) Also, the Indo-Malay region, where the most variation in *Musa* germ plasm occurs (Simmonds, 1962, 1976), is often troubled with 'Bunchy Top' disease (Stover, 1972; Wardlaw, 1972). This disease, thought to be caused by a virus or viroid, does not exist in the Western Hemisphere, and it would be essential to ensure that it is not inadvertently introduced. This is sure to be easier said than done.

In view of all this, the need to use other methods, singly or in combination with conventional breeding, to produce improved bananas and plantains is beginning to be seen as a real possibility (Krikorian and Cronauer, 1982, 1984a,b).

The use of aseptic culture techniques as a breeding adjunct can be envisioned in several ways. They can provide a means of making available ordinarily inaccessible germ plasm. This can be done by virtue of ovule culture for the purpose of in vitro fertilization of embryo sacs with reduced fertility, embryo rescue procedures, or by getting rid of specific pathogens thus enabling more ready introduction of clones from different localities or countries. Multiplication by aseptic culture of specimens ordinarily in limited supply is yet another way of increasing availability. We will touch on these methods but the potential of various other culture techniques, including induction of variation, that might be used in a sort of "combination breeding" program, will be the main emphasis of this paper.

We can do little more here than provide a brief overview of a few of the strategies or possibilities using aseptic culture techniques (Krikorian and Cronauer, 1984a,b; Cronauer and Krikorian 1984a,b).

EMBRYO CULTURE

It was shown as far back as the early 1920s that one could sometimes stimulate growth in aseptic culture, otherwise unobtainable or erratic, of embryos (Krikorian, 1982, and refs. there cited). Since that time, embryo culture has become an accepted and routine procedure for rescuing embryos that would normally not continue their growth into plantlets. Embryo culture from *Musa* seeds has been a valuable adjunct in breeding, and the technique has been useful, for instance, in breeding burrowing nematode-resistant diploids (Rowe and Richardson, 1975,

p. 26). The technique as used now is fairly straightforward but there is a limited published literature. Much still needs to be learned about the culture of embryos (Cox et al., 1960). One facet of embryo culture that does not seem to have been addressed at all involves the possibility of producing triploid (AAA, BBB, ABB, or AAB) plants by culturing and manipulating the normally triploid endosperm of seeds of diploids.

APICAL MERISTEM OR SHOOT-TIP CULTURE

The discovery in the 1960s that excised apical growing points and shoot tips of the orchid genus *Cymbidium* grown in aseptic culture could produce, when appropriately cut, protuberances that resemble normal protocorms that can grow to plantlets, provided the most dramatic impetus for the further development of procedures for multiplying and maintaining plants in aseptic culture (Krikorian, 1982). Shoot tip cultures from many other plants have since been exploited in the acquisition, maintenance, and multiplication of stocks. In some cases, a single plant is generated from one cultured shoot tip; in others, multiple shoots can be stimulated to form (Fig. 1a,b). As long as the development of shoots emerging from the proliferated area of an explanted growing point can be maintained at a rate consistent with their removal by excision, one has an open-ended system. One merely maintains a balance that favors the continued formation of minimally differentiated growths that will organize in culture by adjusting the medium. As these shoots (with or without roots) are removed from the proliferating mass (Fig. 1b) and are transferred to an environment or different medium conducive to further root development (Fig. 1c), new proliferations grow to replace them. In the early 1970s investigators from Taiwan published papers that showed it would be possible to stimulate adventitious buds from banana explants following decapitation of the shoot tip (Ma and Shii, 1972, 1974). These early reports stimulated further investigations, and using the single isolated apex system, Berg and Bustamante in 1974 showed that cucumber mosaic virus-free plants of the 'Cavendish Group' AAA, could be obtained from meristems and lateral buds of virally-infected plants by a combination of heat treatment and aseptic culture. Since then, the culture procedures have been improved considerably, so that we can produce in short order fairly large quantities of shoots and these, in turn, can be rooted in 3-4 days (Krikorian and Cronauer, 1984a; Cronauer and Krikorian, 1984b). The shoot tip multiplication method obviously has great potential for producing multiples of specimens from breeding efforts not ordinarily available quickly and for producing specific pathogen-free planting materials in large numbers. Since they are aseptic, shoot tips can also be used to maintain bacteria- and fungus-free stock for germ plasm exchange, transfer and shipment (Stover, 1977). But virus diseases are more of a problem. Cucumber mosaic virus can be eliminated but there is no certainty that other viruses or viroids can be gotten rid of as well. More work is needed in this area, particularly an effort needs to be made to develop strictly axenic material. There is a good possibility that this could lead to more vigorous plants and increased yields.

From a commercial perspective, the need for rapid clonal multiplication in terms of introducing new clones was, until recently, not seen as a major limitation since export banana cultivars needed to be changed exceedingly slowly and new

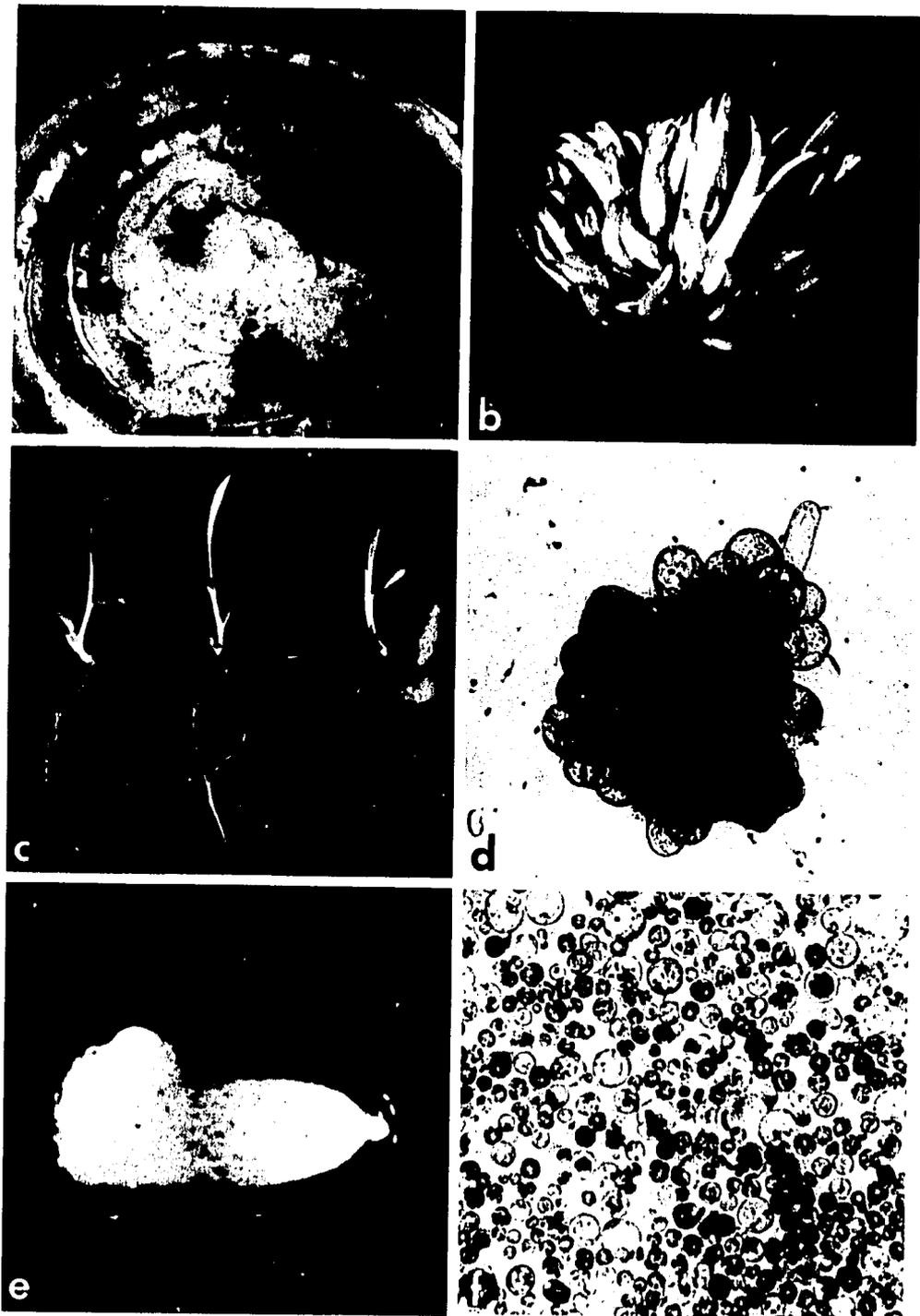


Fig. 1. Photographs of the several levels of aseptic culture technique applied to bananas and plantains: a) growing point of a banana plant exposed through removal of successive leaf bases and ready for excision, 1.3 \times ; b) growth of multiple shoots from a shoot explant stimulated by use of exogenous growth substances. (Many leaf-opposed buds have been released from their normally quies-

industries were relatively scarce. Now, however, there is a need to provide large amounts of clean planting material of newer clones to replace plantations that have been allowed, for one reason or another, to deteriorate. Moreover, there is substantial interest in initiating new large scale plantings.

Ideally, one would like to have excised meristems of many clones and species from diverse geographical ranges maintained in germ plasm storage banks. Here one could maintain germ plasm in aseptic culture under conditions of media composition and/or environment that foster multiplication at the absolute minimal level or even frozen for cryopreservation. When needed, a culture would be removed from the relatively quiescent or frozen state and multiplied as needed by use of the appropriate stimulation sequence of media or manipulation. The feasibility of all this is in real need of being assessed.

MUTATION BREEDING

Using apical meristem cultures

Several investigators have emphasized the benefits that could accrue if a means of producing dessert banana and plantain mutants using aseptic culture techniques could be worked out, applied and the products assessed. The little work that has been done thus far has involved γ -irradiation prior to excision and culturing of the meristem or growing point (Menendez, 1973; Menendez and Loor, 1979; De Guzman et al., 1980, 1982). The field is only now beginning to appreciate that there may be some real potential for bananas. Since the plantlets produced by aseptic meristem multiplication procedures are tiny, we see them as being well suited to mutagenesis manipulation (Broertjes and van Harten, 1978; Gottschalk and Wolff, 1983). The controlled production of variants would be no trivial matter, for somatic variations have contributed greatly to banana diversity. Several scores of mutations, many of which have occurred repeatedly, are known to exist in banana. Several of these have turned out to be economically important, as in the production of the clone 'Highgate,' which is a sport of 'Gros Michel' (Simmonds, 1966). Since the germ plasm base of breeding export dessert bananas on the female side is very limited, i.e., all female parents are 'Highgate,' availability of a variant female germ line could be a very valuable thing. Indeed, if variants of 'Highgate' or 'Gros Michel,' etc., could be produced, the task of breeders might be made easier (Rowe, 1984). Similarly, the production of tetraploids by spontaneous endopolyploidization in diploid culture or by use of colchicine could be useful. We know that multiplication via meristems, *in itself and without mutagens*, does have, under special conditions, potential for producing substantial variability. One cannot be sure, of course, at this time that variability will include resistance to 'Black Sigatoka' disease.

←

cent state.) 1.1 \times ; c) plantlets with roots produced by separation of shoots similar to those shown in (b) and cultured on a root-inducing medium, 0.32 \times ; d) cluster of plantain cells grown in suspension, 58 \times ; e) embryo produced from somatic tissues of a plantain, 10 \times ; f) protoplasts prepared by enzymatic digestion of cultured shoot material, 144 \times .

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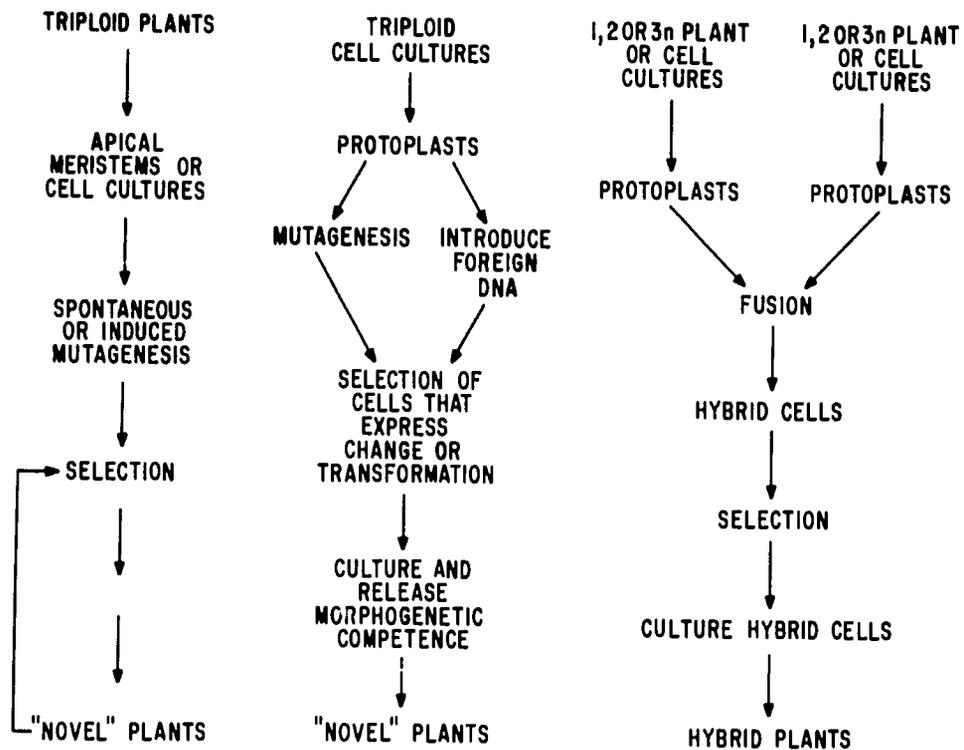


Fig. 2. Schemes for possible use of cell, protoplast and apical meristem culture techniques for producing novel bananas or plantains. See text for details.

Using cell and protoplast cultures

From our perspective, the most challenging and rewarding work is sure to derive from the eventual application of mutation breeding techniques to cells and protoplasts (Fig. 1d,f). This would entail use of cells that have a capacity for production of plantlets from free cell populations (in contrast to organized meristems comprised of many cells). Such cells can grow into plants and are called totipotent. The probability of selecting potentially useful mutations via cell culture techniques would be greatly enhanced since many single cells could be affected and each of these would, theoretically at least, be capable of growing into a plant. The introduction of totipotent cell suspensions of any plant (much less banana or plantain) is generally appreciated as being rather difficult to achieve but we have obtained some encouragement in that we have been able to get structures suggestive of zygotic embryos from *somatic* tissues (Cronauer and Krikorian, 1984a). This strongly suggests that the morphogenetic potential is there but must be controllably elicited (Fig. 1e). Because cell cultures can often be grown in such a way as to produce variation in certain crop plants, certainly vastly greater than that achievable by conventional breeding, one hopes this could one day be coupled with the use of mutagens. In this way the possibilities of mutation breeding via cell suspension culture would be further increased.

Protoplasts, or cells rendered free of their cellulose walls by the use of cellulose-

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degrading enzymes (Fig. 1f), could offer an even more sophisticated means of inducing or even introducing variation into a vegetatively propagated plant such as banana. Ideally, one would prepare totipotent protoplasts, expose them to mutagens and then rear as many of them as possible to entire or mature plants. This is different from cell culture only in that one might be able to start *directly* with cells from the mature plant without isolating and inducing totipotent cells in cell culture. One could also envision, eventually, the possibility of protoplast fusion between triploids and monoploids to make tetraploids from sterile triploids, or diploids with monoploids to produce triploids or even monoploids with monoploids to produce diploids. Having overcome by means of protoplast preparation the barrier to cell fusion inherent in the presence of a cell wall, fusion of protoplasts could lead to fused nuclei between unrelated clones and lead to the production of reconstituted cells from which new plants could grow. Fig. 2 shows how this might be achieved. It also outlines how cells might be modified using mutagens and genetic engineering techniques in combination with protoplast methodology and cell culture.

COMMENTARY

We emphasize that before one seriously anticipates even limited exploitation of any of the ideas presented above or the application of the described methods for practical gains, one must first achieve a more complete understanding of *all of the aseptic culture methods* as they relate to the genus *Musa*.

The facile claims all too often encountered in the lay press and scientific literature alike alluding to the routine usefulness or applicability of the new technologies to a wide range of economically important plants does disservice to the level of the scientific problems that need to be resolved before they are reduced to true technologies. Even so, the attraction of the aseptic culture techniques, as they relate to bananas and plantains, is that useful methods such as meristem and shoot tip culture already exist, and these can go far at present to meeting some of the more immediate needs (Krikorian and Cronauer, 1984a,b; Cronauer and Krikorian, 1984b). Simultaneously, the challenge of the requirement to lay the necessary groundwork for the longer range and more basic problems involving a true genetic engineering using full knowledge of the *Musa* genome must be met.

We have been working towards determining and assessing realistically whether aseptic meristem, cell and protoplast culture techniques have any potential for banana-plantain improvement. We need not go into any detail here (Krikorian and Cronauer, 1984a,b), but we feel that the time has come to make a much more concerted effort on the overall problem. Investigations need to determine the exact parameters for establishment and growth of totipotent tissue, cell and protoplast cultures. We will then need to define the optimal conditions for the challenge of totipotent cells and protoplasts by pathogens, and/or mutagens. Special attention needs to be given to rendering the systems as reliable as possible and to comparative studies using a wide range of clonal materials or biotypes anticipated as suitable for such challenges.

A controllable system capable of yielding entire plants from absolutely single cells or protoplasts is an essential and crucial feature of the genetic engineering technology. Without this capability, there is no reliable means of producing and/

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or selecting tolerant/resistant new germ plasm. Meristem culture has a real place and is potentially valuable but it cannot substitute for the cell and protoplast culture systems. Whether or not the opportunities to develop anew or further any of the aspects of the culture systems outlined will be seized, remains to be seen. Even so, it is hoped that readers will now have a better appreciation of where the real problems lie. Readers should also recall that the kinds of tissue culture problems considered here for bananas will apply to virtually all plants.

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Somatic Embryos from Cultured Tissues of Triploid Plantains (*Musa* 'ABB')

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ABSTRACT

Somatic embryos of two triploid plantain clones ABB 'Saba' and 'Pelipita' were produced in liquid from cells derived from multiplying shoot cultures. The medium consisted of a modified Murashige and Skoog (1962) medium with the auxins 2,4-D (1 mg/l) and 2,4,5-T (1 mg/l) and the cytokinin BAP (0.1, 1 mg/l). Whereas proembryonic masses formed in each instance, green somatic embryos formed only in those treatments containing 2,4,5-T.

INTRODUCTION

Cooking bananas or plantains are a major food staple in many parts of the tropics and subtropics. Along with the dessert bananas, they compose one of the world's most conspicuously sterile crops. The clones of commerce are triploid and, for all practical purposes, seed sterile. Therefore, propagation is necessarily by vegetative means. Efforts to improve both bananas and plantains using conventional breeding techniques over the past sixty years have produced very limited results (Rowe and Richardson 1975; Rowe 1984). Consequently, attention has been drawn to the possibility of using aseptic culture techniques to induce variation and to recover novel plants (Krikorian and Cronauer 1983a). To date, the majority of work has focussed on the multiplication via stem tip cultures (e.g. Ma and Shii 1972; Vessey and Rivera 1980; Bower 1982; Dore Swamy et al. 1985; Krikorian and Cronauer 1985). There has been no published report so far as we are aware of the establishment of morphogenetically competent cell suspensions. Roots have been reported, however, from callus cultures derived from inflorescence axis of a dessert banana, AAA 'Robusta' grown on semi-solid medium (Srinivasa Rao et al. 1982).

We describe here the production in liquid of somatic embryos from cells derived from cultured shoots of two plantain clones, 'Saba' and 'Pelipita'. These are members of the starchy 'ABB' group which have been attracting considerable attention because of their high degree of tolerance to Sigatoka Leaf Spot, 'Sigatoka Negra', caused by *Mycosphaerella fillicensis* var. *difformis* (Stover 1980).

MATERIAL AND METHODS

Multiplying shoot cultures of the *Musa* ABB plantain clones 'Saba' and 'Pelipita' were established according to the following procedures. Shoot tips (meristem plus 1 or 2 leaf primordia) were isolated from suckers 1.5 m tall which, after surface sterilization, were cultured on semi-solid medium of the following composition: the mineral salts of Murashige and Skoog

(1962), 100 mg/l inositol, 4% (w/v) sucrose, 1 mg/l thiamine HCl, 5 mg/l BAP, adjusted to pH 5.8 and solidified with 0.7% Difco Bactoagar. Multiplying shoot cultures were induced by splitting young shoots longitudinally through the apex. Established cultures were routinely subcultured every 3 to 4 weeks (Krikorian and Cronauer 1984). Small clusters of 5 to 8 shoots were separated from large multiplying clusters with scalpel incisions and transferred to 125 ml erlenmeyer flasks containing 15 ml of liquid medium of the following composition: the mineral salts of Murashige and Skoog, 100 mg/l inositol, 2% (w/v) sucrose, 5% (v/v) coconut water (CW), and 1 mg/l thiamine HCl, adjusted to pH 5.8 (hereafter designated BMS). Three hormone treatments were used: 1 mg/l 2,4,-D and 1 mg/l BAP, 1 mg/l 2,4,-D and 0.1 mg/l BAP, and 1 mg/l 2,4,5-T and 1 mg/l BAP. The flasks were placed on a rotary shaker at 80 rpm in a room maintained at 50°C ± 2°C, 50% RH and on a 16:8 L:D cycle (10.2 x 10³ lumens/m²).

Eight days later, the 'Pelipita' cultures were transferred to fresh medium as follows. Shoot pieces were removed from the flasks and swollen shoot clusters were cut along their natural lines of separation. Blackened tissue was removed as necessary. The tissue was re-inoculated into 15 ml of fresh medium of the same composition in a 125 ml erlenmeyer flask. Any cells which had sloughed off into the culture medium were allowed to settle and were transferred with a pipet to the new flask in 2 to 5 ml of the old medium. This procedure was repeated 26 days later. The 'Saba' cultures were transferred to fresh medium once, 24 days after they were initiated. When this procedure was repeated for both clones 9 days later, the cells and small cellular masses which had sloughed off into the liquid were transferred to "T" shaped culture tubes (Caplin and Steward 1949) which contained 10 ml of culture medium. The T-tubes were rotated at 1 rev/min on an apparatus modelled after that first described by Steward et al. (1952). Embryos were removed from the T-tubes 12 to 14 days later and placed on semi-solid medium composed of BMS + 5 mg/l BAP without CW.

RESULTS AND DISCUSSION

When shoot clusters were placed in liquid medium, tissues became swollen and absorbed much of the medium. This was presumably due to cell expansion. At the time of the first transfer to fresh medium, the liquid medium contained cells which had sloughed off the enlarged tissue pieces. Microscopic examination of these cells showed that they were for the most part elongated single cells. Occasionally, clusters of



Fig. 1. Some representative stages of somatic embryogenesis in *Musa*.

a. White nodular masses derived from the culture in liquid of 'Pelipita' shoots. Masses such as these could yield somatic embryos in 12 to 14 days ($\times 2.25$); b. Somatic embryos of 'Saba'. Note the relative uniformity of embryo development. Even so, one shows twinning of the embryonal axis (arrow 1) and one shows bifurcation of the root axis into two independently growing root tips (arrow 2) ($\times 3$); c. Close-up somatic embryo of the clone 'Pelipita'. Note the nascent shoot axis on the left ($\times 6.2$).

more compact cytoplasmically denser cells were observed. When the cultures were transferred, small white proembryonic masses were observed floating free in the liquid (Fig. 1a) and growing on the shoot clusters, both at the shoot bases and along the leaf sheaths. The free floating masses were transferred to T-tubes along with the free cells. Twelve to 14 days later, in both clones, green somatic embryos were observed in cultures containing 2,4,5-T (Figs. 1b,c). In the other hormone treatments, the white masses grew in size and their number increased but none of them yielded embryos. Those embryos which were placed on semi-solid medium went on to produce long roots. However, shoot development was not observed. When nodules which had formed in the 2,4-D treatments were transferred to BGS, they failed to develop into embryos.

The production of proembryonic masses and their subsequent growth and development into somatic embryos suggests that the genes which control embryo formation had been activated during the culture process. Although proembryos were produced in all treatments, further development to readily recognizable green embryos with a shoot-root axis was limited only to those treatments which contained 2,4,5-T. While this is similar to those situations where proembryos can form in the presence of exogenous auxin, as in the Gramineae (Ho and Vasil 1983), it is in direct contrast to those situations where the level of exogenous auxin must be lowered or eliminated before somatic embryo formation or organization occurs (cf. Tisserat et al. 1979; Krikorian and Kann 1981; Wernicke et al. 1981). It also suggests that under our conditions the required activation could not proceed or was incomplete in the absence of exogenous 2,4,5-T. Work not reported here shows the nature of the sequential effects to be elusive and that the regulation of the process described is not well enough understood so as to be strictly controllable. Although the kind of morphogenetically competent cell suspension which has frequently been established from many dicotyledons but far less often from monocotyledons has not yet been obtained, we have shown that somatic embryos can be produced. This is all the more remarkable because the clones are triploid.

Because the diploid bananas *M. acuminata* (AA) and *M. Balbisiana* (BB), both of which are ancestral "parents" of these plantain clones (ABB) are seeded and studies of their seeds, particularly that of *M. Balbisiana* have been made, we are able to draw some comparisons between the somatic and zygotic embryos.

Although the somatic embryos depicted here are larger than diploid embryos isolated from seeds (4 mm vs. 2 mm), the fidelity of their overall structure and conformity to Scitamineacean embryos is noteworthy (Gatin 1908; McGahan 1961; Bouharmont 1962-3). The capacity of this and analogous culture procedures to produce reliably and predictably morphologically and anatomically correct somatic embryos which can develop into young rooted plantlets is currently being assessed.

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NEW RESEARCH POTENTIALS FOR MUSA IMPROVEMENT:
THE REALITY AND THE CHALLENGE*

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*This paper is a brief summary of an extensively illustrated presentation made in San Jose, on 22 September 1985.

ABSTRACT

Methods are reasonably well worked out which permit germ plasm to be multiplied rapidly in a disease-free or specific pathogen-free state from excised shoot tips or meristems (both vegetative and floral) of various Musa clones. This in itself may provide a means of generating somaclonal variants or spontaneous mutants and there is an urgent need to evaluate this potential. But one can prophesy with some confidence that the most controlled means of producing novel genotypes and phenotypes will emerge from procedures using free cells. Development of cell culture methods for the eventual large scale production of mutants from somatic tissue is a necessary prelude. Musa clones provide a challenging test system through which one can at once investigate both morphological and physiological pre-requisites for expression of morphogenetic competence under aseptic culture conditions. Such studies should also enable one to develop the protocols that will be necessary for the free exploitation of culture procedures. These, in turn, will provide the base from which investigations on the development of disease-tolerant clones can depart.

INTRODUCTION

The "new biotechnologies" are being heralded as offering the possibility of realizing the broad scale solution to the world's agricultural (and health) problems. Like other new trends, it is often difficult to separate possibility from achievement and expectation from actuality. There is no doubt that the new technologies offer powerful tools for investigation and, for the moment at least, it is in that context that people interested in the wide scale growing and cultivation of bananas and plantains should view them.

This overview of research opportunities is intended to draw attention to some of the more recent developments. It is not intended as an exhaustive documentation of each of the developments. Literature citation is not attempted in the text. Instead, a list of papers from my laboratory is provided. These will permit full entry and access to the relevant literature.

WHAT ARE THE EXPECTATIONS?

Some time ago I attempted to prophesy the kinds of achievements that might be possible from biotechnological developments if one saw them from a short or near-term, intermediate-term and long-range or distant-term perspective (Table 1). These possibilities are impressive and if they could be routinely applied "across the board," so to speak, to a wide range of species or economically important plants, the impact would be staggering. As it turns out, many of the possibilities remain just that -- possibilities. Even now there may be some exceptions to my attempted categorization and some major breakthrough could change all of it, but a conservative assessment such as that given in Table 1 is certainly justifiable at this point in time.

One of the many major requirements for the biotechnologies to be implemented, even on a relatively straightforward scale for Musa (or any other

plant for that matter) involves the availability of a range of aseptic culture techniques. These include ovule culture, pollen culture, embryo culture, apical meristem or shoot tip culture, callus culture, cell culture and protoplast culture. Without these methods, there will be relatively limited opportunity to do the necessary investigations that could lead to the generation or "engineering" at will of some novel and highly desirable banana and plantain clones. No new "miracle banana" or plantain can be possible without the foundation techniques. And, the fact of the matter is that although we are improving and expanding our techniques for Musa, we are some distance away from the controlled use of the cell and protoplast methodologies. This means in effect, and from a practical perspective, we are, in the short term, restricted to the use of meristem or shoot tip culture "technology".

It is also important to emphasize that the aseptic culture techniques, unless proven otherwise by achievement, are best viewed as adjuncts and supplements to existing breeding schemes. They should not, certainly in the short or intermediate term, be seen as replacements for the more conventional or traditional genetic improvement approaches. Nevertheless, one should not lose sight of the fact that there is no banana or plantain clone in commercial or in widespread production anywhere, that is human-generated. All edible Musa resources are products of natural selection. This in itself emphasizes the difficulty of the problem of breeding an essentially seedless or infertile crop, or it emphasizes the narrow perspective from which most breeding schemes, up until recently at least, have had to operate. Commercial, for export, dessert bananas intended for the markets of North America, Europe, Japan etc. have very stringent, exacting requirements in terms of certain agronomic qualities, ripening and post harvest physiology characteristics etc. This and problems of fertility (or should I say infertility?) has restricted

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the genetic improvement approach to the use of 'Highgate' (AAA) as the female breeding parent. Unless one can think of ways to enhance the variability of 'Highgate', one is highly limited right from the outset. In short, there is considerable reason for thinking that some kind of adjunct or supplementary approach to sexual hybridization is justified and indeed might be useful. In the very least, it can broaden our scientific base of knowledge on this very import group of plants. It seems appropriate that the mentality of "crisis management" in terms of banana and plantain problems be supplanted with a more enlightened one of "the more we know about the basic biology and biochemistry, the better off we are when confronted with a particular difficulty".

SHOOT TIP AND MERISTEM METHODS

Shoot tip methods were first reported in 1972 by Ma and Shii from Taiwan. Berg and Bustamante published in 1974 a paper in which they reported utilizing their interpretation of the technique to produce cucumber mosaic virus-free plants. From what I have been able to discern, the United Fruit Company in La Lima, Honduras and DelMonte in Mindanao, Philippines integrated the shoot tip methodology fairly early on into their research and production programs to a greater or lesser extent. In Taiwan, the technique, somewhat modified from that published, became a mainstay of a planting and production program that is geared primarily to the needs of the rather distinctive Japanese market. In the Philippines, Emerita de Guzman and her associates utilized the shoot tip technique to special advantage in their work on abaca (Manila hemp, Musa textilis) but shoot tip culture was also extended to edible bananas as well.

Scattered general reports in diverse and generally inaccessible, or localized more-or-less industry-gearred literature seems to have restricted interest in banana and plantain tissue culture*. Had there been a conscious effort to publish in the broader plant physiological-plant tissue culture-

botanical literature, there would have been a greater interest in developing the tissue culture procedures.

An important point of principle emerges here. In my view, the tendency for secrecy and the proprietary attitudes of industry are self-defeating in the long run. Had "the Industry", or those with ties to industry not exhibited such a high level of confidence in the then existing shoot tip culture methodology or "technology," they would have seen the urgency to follow through on that which was obvious to those familiar with tissue culture. Namely, it had to be asked to what extent and with what level of confidence could one view the methodology being used as commercially viable, cost effective and dependable?

Shoot tip methods are impressive when one considers that a single isolate of the vegetative shoot growing point can yield several million plants in a year (cf. Fig. 1). It is also impressive if one considers that the floral meristem (the male bud growing zone) is equally responsive and can be used as a source of explant material for multiplication.

There is every reason to believe that the tissue culture multiplication methods can probably play a major role in enabling reliable

- rapid expansion and availability of new clones and hybrids
- production of disease-free or specific-pathogen-free planting material for new areas

Footnote:

* My own interest in Musa tissue culture, and that of the late Dr. de Guzman dates back to contact with Professor F. C. Steward at Cornell University. In that period (late 1950's to early 1960's) there was much basic research supported by United Fruit going on in that laboratory with 'Gros Michel'.

- germ plasm shipment and exchange
- production of plants for research purposes including testing against challenge of disease, insects, nematodes; use in nutrition and other physiological studies.

The shoot tip methods also hold promise for genetic conservation of germ plasm and gene bank maintenance.

The word "probably" is deliberately inserted above for the following reasons. We still do not know the extent to which one can infallibly rely on the "technology" from the perspective of the "clonal fidelity" of the plants produced from meristem or shoot tip culture. Field testing has been generally limited and only recently has information begun to emerge on relatively small scale trials (see these ACORBAT proceedings). I have attempted over many years without success to get aseptic culture-generated plants field tested. The arguments against field trials have been the cost, and more significantly, the view has been repeatedly expressed that nothing "new" could emerge since the shoot tip culture technique is "well established" and "routine". Nothing is further from the truth!

There has been information for quite a few years that plantlets of many species produced via "tissue culture" are not necessarily true to type. The changes encountered may be rather extensive and seem to vary according to the plant and the procedures used. There is very little information on the causes of the variation (Table 1). The changes are frequently "lumped" under the all-inclusive designation of "somaclonal" variation. In general, the more organized the initial explant, the less the variation. The less organized the culture starting material, the more the variation. On this view, meristems and shoot tips would produce less variation and callus, cell suspension or protoplast procedures would generate the most varied plantlets. Even so,

substantial variation can be and has been encountered in some shoot tip generated materials in several plant species other than Musa. Since Musa in particular is a genus that has shown considerable potential for variation and mutation, it seemed reasonable to me at the outset that variation could emerge from tissue culture. Indeed, as many or more examples of useful mutations being grown on a large scale could be cited for banana and plantains than perhaps any other cultivated plant.

There is every reason to believe that the challenge and opportunity exists now to undertake careful studies to ascertain, once and for all, whether useful and practical variants can be produced by shoot tip culture. The encouraging results obtained by S. C. Hwang and his colleagues in Taiwan, at the Banana Research Institute, Pingtung, with 'Cavendish' clones by the use of shoot tip culture-generated plants to screen for Panama Disease or Fusarium wilt race 4 resistance (see this Volume) should serve to stimulate and increase efforts in this direction.

POSSIBLE STRATEGIES FOR THE NEAR-TERM

It is imperative that carefully co-ordinated studies which correlate aseptic culture technique and procedures and the field performance of tissue culture progeny be initiated. Indications of variability of tissue culture generated banana (and to a much lesser extent plantain) plants are indeed starting to surface or accrue but a feature which is conspicuously lacking (from the perspective of a tissue culture expert) is the lack of details on such things as the exact nature of the culture used, the origin and size of the explant, the duration of various phases of the culture, the manipulations the material was subjected to etc. etc. All these details have very real implications for stability. As I, and many others in the field, have put it, "there is tissue culture and there is tissue culture". The time has come to

carry out specific field tests, using aseptic culture-generated material of specifiable heritage and lineage. This should be done using the several methodologies now accessible through various laboratories engaged in Musa tissue culture. Assuming it can be established by this means which of "the methodologies" or "protocols" is best suited for production of stable material showing high genotypic and phenotypic fidelity, one can rigorously specify and enumerate the procedure for all to follow for this purpose. Similarly, if it can be disclosed that other, equally well definable, procedures and protocols yield great variability, then these would be avoided in the event stable multiplication is desired. Also, it would be critical to avoid those methods for germ plasm storage or in connection with cryopreservation procedures since stability is absolutely critical.

If one can rely to any extent on the ability to elicit variation by any feature of the aseptic culture procedures per se, then the value of this capability will perhaps be best realized in conjunction with screening for tolerance or resistance to specific pathogens, nematodes, herbicides etc. Huge numbers of aseptic culture-generated propagules could be challenged at the laboratory or nursery level to select out those that might show desirable qualities. While somewhat later stages of testing would absolutely require field trial, it is important to realize that a field trial method for primary selection of tolerance or resistance would be too time-consuming and costly to implement. One cannot over-emphasize the skill and expertise that will be required to disclose more subtle features in tissue culture-generated plant populations. Only those very familiar with the crop and a particular variant of it will be able to detect such possible changes as yield and ripening characteristics of fruit, taste etc. Perhaps even such changes as increased fertility will be detectable. All the above is said in the spirit that we all

have an opportunity now to enter upon a new period of investigation and research. One should not, until it is proven beyond all doubt, assume that shoot tip or meristem culture generated bananas or plantains are strictly clonal. It would be a great advantage to determine what provides for stability; what provides for change.

TRANSFERRAL OF TECHNOLOGY

The argument has been made by some that the new biotechnologies will be difficult to put in place in less economically developed countries or countries in development in the tropics and subtropics. While it is true that it may be somewhat easier to achieve in situations such as those encountered in North America and Western Europe, I personally do not see any difficulty in securing the expertise to do banana and plantain tissue culture work in the tropics, especially as it relates to shoot tip and meristem procedures. There is proof that this can be done and the laboratory of Dr. Ludwig Muller of CATIE in Turrialba, Costa Rica is but one example; another is the laboratory of Drs. Antonio Angarita Z. and Margarita Perea Dallos of Universidad Nacional de Colombia, Bogota. My laboratory and theirs have been and are co-operating on several projects.

As more work is done throughout the world, a major challenge will be to so co-ordinate efforts dealing with tissue culture methodology and evaluation of tissue culture progeny, that all the minutiae of detail are kept straight and communication is clear and unambiguous as what is being done by whom. If a project is to succeed, co-operation and good faith are essential on the part of both scientific investigators and the grower industry - be a project on a modest or large scale.

The transferral of technology as it relates to a precise micropropagation protocol from a laboratory setting to the banana and plantain industry will

require yet another set of standards for co-operation. The work will be labor-intensive and skill-requiring. The economics of micropropagation will have to be analyzed and it will have to be established what will be the proper place for it in any given special setting. Quality control procedures will have to be implemented as well. As an example of quality control, one can use the question of sanitation and microorganism-free culture production. Indeed, it would be highly desirable to have completely axenic materials (virus, mycoplasma-free etc). It cannot be assumed that "aseptic" culture procedures, necessarily generate "germ-free" plantlets. Unless special and stringent precautions are taken, one is not guaranteed of clean material. One may in fact, inadvertently spread contamination even as one multiplies propagules. We have tested a number of cultures from a number of sources and have detected microbial contamination in cultures that were nominally "clean". All this shows that the "new" technologies present their own problems and one will have to become attuned in a more sophisticated way as to what is implied when one talks about the various aseptic culture techniques.

MORE "ADVANCED", LONG-TERM OPPORTUNITIES FOR RESEARCH

I will not go into detail here on the more long term opportunities for research. The select bibliography (arranged chronologically) will provide access to the relevant literature. Suffice it to say that the more long term benefits such as those outlined in Table 1 will only be achievable when the cell and protoplast techniques become routinely and predictably usable. Since bananas and plantains have not yet proven themselves to be "easy" plants to manipulate by these techniques, one can predict that more basic research work will be necessary. We have been very cautious in our expounding the possibilities for research using the new biotechnologies. It does a disservice to the level of the problems to be faced and the limitations of our

present knowledge to make it appear that developments needed for Musa are around the corner.

It seems especially appropriate to emphasize, finally, that the problems of dessert banana breeding and plantain breeding provide their own special set of challenges but in principal they are the same. International agencies, presumably fearful of criticism that they should not fund research projects that seemingly benefit the huge transnational companies concerned with the export dessert banana trade, have thus far seemed anxious to disassociate themselves from efforts to breed bananas. The argument is that plantains and cooking bananas are food staples for the poor, including the subsistence farmer. But it should be recalled that plantain exportation has also become a big business. It will be interesting to see the outcome of breeding efforts to produce bananas and plantains required for circumstances other than for export. Less demanding, more local needs that do not have to take into account the almost hopelessly tight guidelines that face the commercial, export, dessert banana breeder are sure to be more manageable for those using the plant breeding genetic improvement approach. It seems arbitrary to provide support for plantains and cooking bananas and no support for bananas. Dessert bananas figure heavily in the economy of all producer countries whether intended for export or not.

Controls which are, in my view at least, out of the hands of the scientists are the only method by which all of the people of the "banana and plantain world" will be able to share in the anticipated benefits of the positive impacts of the new biotechnologies that one hopes will emerge in the not too distant future. Science feeds on itself. One advance leads to another. There seems no justification to limiting our horizons by branding one type of research "good" and another "bad". The real problems or

constraints to the kind of progress envisioned are not, however, only restricted to financial resources. They include shortage of investigators and support personnel with adequate training and scientific background; a need for a bureaucracy sympathetic to basic research and the need for more administrators able to facilitate implementation of programs; need for adequate release time for investigators in universities from lower level undergraduate teaching and, last but not least, the need to recognize not only the capabilities but the limitations of the various culture and/or biotechnology techniques. It goes without saying that teamwork at the individual, institutional, national and international level is important. Similarly, there must be maximum integration with other disciplines. All agree that there must be frequent and open communication. If we can abide by these guidelines, one can safely guess that undreamed-of opportunities will emerge for improvement of one of mankind's most important vegetatively propagated crop plants.

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Table 1. Potential Benefits from the "New Biotechnologies"

RESEARCH WITH POTENTIAL FOR NEAR TERM IMPACT

ASEPTIC CULTURE TECHNIQUES CAN FACILITATE:

- Rapid multiplication of select specimens
- Elimination of virus and specific pathogens
- Virus indexing
- Germplasm introduction and evaluation
- Germplasm collection, preservation and management
- Production of polyploids, haploids, somaclonal variants for new crop production and use in breeding, etc.
- Elimination of certain breeding barriers
 - . in vitro fertilization in ovulo
 - . embryo rescue and/or storage
 - . androgenesis
 - . gynogenesis

RESEARCH WITH INTERMEDIATE IMPACT

- All the above in more recalcitrant species, plus
- Selection for complex traits such as tolerance to stress
 - . biotic - diseases and pests
 - . abiotic - temperature
 - salt
 - herbicides
- In vitro mutation breeding
- Cryopreservation

RESEARCH WITH LONG RANGE IMPLICATIONS

- All the above in still more recalcitrant species, plus "Genetic engineering"
 - . transformation by selectable genes etc.
 - . organelle transfer
 - . wide crosses - somatic hybridization
- Understanding controls in developmental and physiological processes

Table 2. Possible Causes of Somaclonal Variation

- o Specific karyotype selection from mosaic, chimeric and polysomatic tissues and plants
- o Karyotype changes due to differential response to culture procedures (media composition and/or environment)
 - non-disjunctional aneuploidies in culture
 - mitotic arrest leading to polyploid lines
- o Somatic gene rearrangements or mutations of the karyotype
- o Gene amplification or diminution
- o Virus elimination from selected lines of a culture
- o Rearrangements or mutations in organellar genomes
- o Altered nucleo-cytoplasmic interactions resulting in regulation changes
- o Sudden reorganization of the genome by transposable elements
- o Variegated position effects of chromosomal rearrangements (inversions, translocations etc.)

Fig. 1. Left, photograph of a shoot tip system showing multiple branches; right, beakerfull of shoots subdivided from such clumps but destined to be discarded after a routine sub-culture procedure has been carried out. The number of shoots available for manipulation are so enormous that extra propagules sufficient to generate a plantation are regularly thrown out.

Aseptic Culture Methods for Plantain
and Cooking Bananas

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Key Words: Musa tissue culture, suspension culture, protoplast culture.

ABSTRACT

Methods are now reasonably well worked out which permit germ plasm to be multiplied rapidly in a disease-free or specific pathogen-free state from excised shoot tips or meristems (both vegetative and floral) of various Musa clones. This in itself may provide a means of generating somaclonal variants or spontaneous mutants and there is an urgent need to evaluate this potential. But one can prophesy with some confidence that the most controlled means of producing novel genotypes and phenotypes will emerge from procedures using free cells. Development of cell culture methods for the eventual large scale production of mutants from somatic tissue is a necessary prelude. Musa clones provide a challenging test system through which one can at once investigate both morphological and physiological pre-requisites for expression of morphogenetic competence under aseptic culture conditions. Such studies should also enable one to develop the protocols that will be necessary for the free exploitation of culture procedures. These, in turn, will provide the base from which investigations on the development of disease-tolerant clones can depart.

I believe there is considerable potential for safeguarding the world's plantain and cooking banana resources, and for plantain and cooking banana improvement by the use of modern meristem, tissue, cell and protoplast culture techniques. These range from methods for rapid, clonal multiplication of specific pathogen or disease-free material from shoot tip or meristem cultures on the one hand, and the generating of useful genetic variation on the other hand. I will emphasize at the outset, however, that I do not see the aseptic culture techniques as replacing more traditional methods and approaches. Instead, one sees them as supplementing them and being used in combination. At best, they can "go hand in glove". Although Champion (1963), Stotzky et al (1964), deLanghe (1969), Menendez (1973), Broertjes and van Harten (1978) and others apparently early envisioned mutagenesis of suckers as a method of banana-plantain improvement, the means whereby this vision could be even attempted has only relatively recently come to light.

There is an increasing body of published literature on the shoot tip and apical meristem culture of bananas and plantains. While there apparently was no particular pre-occupation in the publication and dissemination of the specifics needed to do this, we have seen to it that detailed protocols are now available and this should go far towards permitting investigations and technicians to achieve "state of the art" capability (see Krikorian and Cronauer, 1984a; Cronauer and Krikorian, 1984a and b and in press).

The vast majority of effort insofar as stem tip culture is concerned has, at least on a world wide basis, perhaps been placed on AAA or dessert banana clones. Work in the Stony Brook laboratory on both AAA, AAB and ABB¹

¹Saba is now described as a BBB genotype (Valmayor et al., 1981).

clones has shown that there is considerable variation in response in culture due to genomic differences in sucker production. Our findings with the cooking bananas 'Pelipita', 'Saba', 'Gana Auf', and the plantain 'Harton' indicates that the multiplication rates vary substantially (Cronauer and Krikorian, 1984a). Fortunately, most responses are more than adequate in terms of material producible in aseptic culture.

A potentially important recent development at Stony Brook has been that we now know that the same kind of strict meristem system achievable with vegetative apices can be initiated from floral apices (Cronauer and Krikorian, 1985). This work has shown that the apical dome of flowering Musa seems not to be irreversibly determined and that it can be stimulated to embark upon a growth mode that is vegetative rather than floral. It has given us a valuable procedure whereby we can manipulate, relatively easily, the size of the initial apical explant. The vegetative apex is much more difficult to work with since it is buried deep within the corm and does not, therefore, readily lend itself to removal, with any degree of confidence, along with controlled amounts of subjacent or lateral meristematic tissue. In contrast, the meristematic region of the floral apex is more prominent and protrudes in such a way that it can be excised precisely (Cronauer and Krikorian, 1985). This may well prove useful in achieving specific pathogen-free material whether from viruses, viroids, mycoplasmas etc. It provides the attractive option of multiplying a clonal population without upsetting the normal sucker production sequence of a new clone which is available only in limited supply.

The means to establish whether bacteria and fungi-free materials have been produced in a tissue culture setting are more or less straightforward to implement. Testing by specific nutrient broth for bacteria and fungi has

been followed routinely in this laboratory and has long been practiced by responsible tissue culturists concerned with mass propagation. Provided precautions are taken and monitoring is rigorously exercised, problems can be handled. No attempt will be made here to deal with the challenge posed by the situation of ascertaining incontestably that materials are specific virus or viroid free.

I have provided a scheme which need not be republished here (cf. Figure 2 of Krikorian and Cronauer, 1984b) that raises the very real possibility of shoot or meristem culture procedures being used to generate or select spontaneous mutants or somaclonal variants of Musa clones. To do this one would need to expose very large populations of regenerated plants to pathogen challenge. It would be a major (but by no means impossible) undertaking, but there is no doubt that it would be a worthwhile effort. One of our problems at Stony Brook has been the inability to convince people who have field facilities to test large populations of aseptically generated plantlets in locations known to be troubled by pathogens. (Hopefully this will change). Even so, there is no doubt in my mind that the chances of selecting potentially useful spontaneous induced mutations via cell culture techniques could even more greatly improve retrieving disease-tolerant lines of plantains and bananas, since plantlets could, in theory at least, arise from single cells in virtually unlimited numbers. The most challenging and rewarding work involving tissue culture techniques would in my view entail initiation of suspensions (in contrast to meristem, shoot tip, tissue or callus cultures) that have a capacity for production of plantlets either by initiation of shoot and root growing points adventitiously on a callus derived from cells, or by the formation of adventive or somatic embryos. The latter would be highly desirable from both the view point of either

micropropagation, somaclonal variant production or "mutation breeding" (see Krikorian, 1982).

If one subscribes to the view that a basic cell biology approach holds great promise for a long term solution to various physiological or pathogenesis problems of plantains and bananas, an early priority is to develop cell methods. There is much evidence that can be cited to demonstrate conclusively that tissue culture methods in the broadest sense of the word are capable of producing very useful variations and mutations. I believe that we will be able to apply tissue culture to select or generate new variations of plantains-bananas which are tolerant to both 'Black' and 'Yellow Sigatoka', and perhaps 'Panama Wilt Disease'. Efficient capabilities for screening and selection of disease tolerance or resistance is impossible, however, without the effective development of a wide range of aseptic cell culture methods.

Induction, at will, however, of totipotent cell suspensions of any plant (much less plantains or bananas) is generally appreciated as being very difficult to achieve. It is not simply a matter of the extension of existing "technology"; it is not a "routine matter." It is a basic research problem. From time to time we have directed efforts to probing the potential for working with cell suspensions of various Musa clones. We have also done some studies with protoplasts (see Cronauer and Krikorian, 198). The results with each of these systems, which we need not elaborate here, have underscored the generally recalcitrant nature of the genus. The progress with cell suspensions culture has been slow when candidly evaluated in terms of real practical potential. We have, however, been able to induce from the 'Pelipita', Saba', and 'Harton' the formation of structures substantially like somatic embryos. These structures can form roots but the shoot

development is poor and generally arrested (Cronauer and Krikorian, 1983). More recent efforts to induce new growth forms or to initiate different modes of growth from aseptically cultured materials have disclosed scientifically interesting results but the demonstration that these will have any implications for immediate practical goals await further work (Krikorian et al, unpublished; Cronauer and Krikorian, unpublished.) This is not the place to go into a detailed discussion of our findings but one can perhaps generalize with the following.

A main priority must be to develop further and to render new cell culture systems for Musa as reliable as possible (see Fig. 1). This means probing the systems carefully and systematically. A considerable store of knowledge has been accumulated by us and others. The clones on which studies should concentrate would include those suspected or known to be good breeding parents, as well as those like the 'Horn Plantain' which is susceptible to Black Sigatoka and which is highly prized as a good food source. Similarly, 'Saba' and Pelipita' are examples of Sigatoka-tolerant cooking bananas which have many good qualities but could be vastly more useful if change in other less appreciated characteristics could be brought about. The rationale here is that it would be best to work with clones with good qualities that would require relatively little, but nonetheless critical modification to render them useful (Shepard, 1980; Gottschalk and Wolff, 1983). However, as wide a range of clonal materials or biotypes as necessary should be investigated based on the knowledge of the potential they would have for disclosing principles of regeneration in culture, or stability and instability of genotype, or of conferring tolerance or resistance to various clones. Basic, scientific information must be obtained on how to use the tool of tissue culture for the type of problems faced by those seeking to improve plantains,

cooking and dessert bananas. This will not be achieved overnight. The new technologies offer no controlled solutions. The empiric progress has been encouraging and the impetus of research must not slide.

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I am grateful to the U.S. Agency for International Development for making possible my presence in Abidjan. The research work and views presented here, derive from efforts supported by U.S. Agency for International Development and the U.S. National Science Foundation-Division of International Programs. Special recognition is made here also of the contributions made by Ms. Sandra S. Cronauer, Mary D. Scott and David L. Smith.

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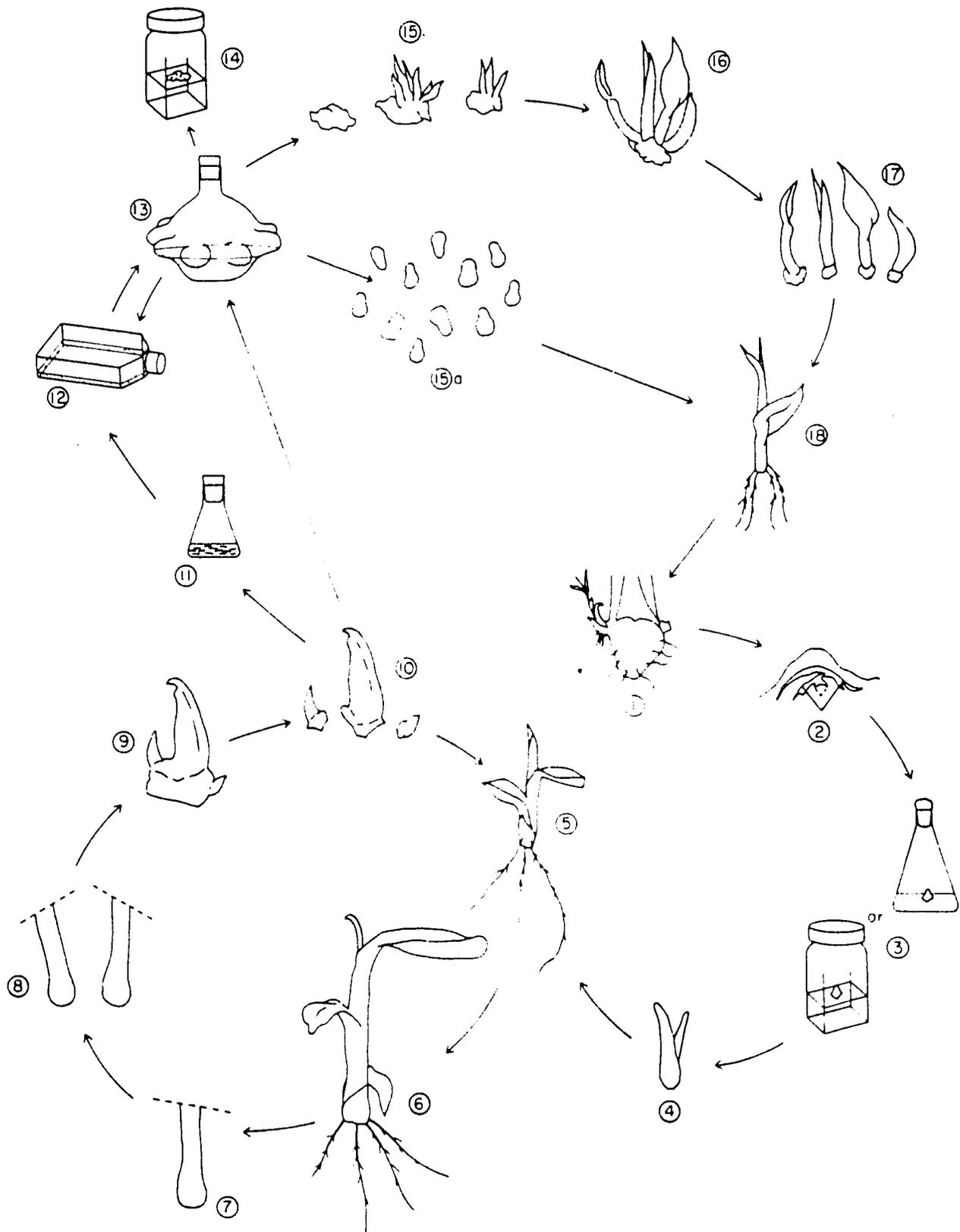
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Fig. 1. Diagrammatic representations of the procedures followed in the establishment of aseptic shoot apex or meristem cultures (1 through 3) and the formation of rooted plantlets (4 and 5). Multiple branching cultures can also be produced (6 through 10). Somaclonal variation (spontaneous change or mutation) or challenge with mutagens have promise to produce tolerant variants but work with free totipotent cells or protoplasts, theoretically at least, would provide the greatest chance for controlled, successful changes being introduced. We are currently investigating the isolation and regeneration of protoplasts and cells from our cultured material (11 and 12). This scheme includes developing procedures for the regeneration of protoplasts to produce suspension cultures, callus, and plantlets (13 through 18) or ideally, somatic embryos and plantlets (15a and 18).



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HANDBOOK OF PLANT CELL CULTURE,

Volume 2

Crop Species

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SECTION VI

Tropical and Subtropical Fruits

CHAPTER 12

Banana

A.D. Krikorian and S.S. Cronauer

HISTORY OF THE CROP

Bananas are one of the earliest crops cultivated by man and, for the most part, only clones derived from natural evolution are cultivated for food. The extensive work of Simmonds and Shepherd (1956) suggests that edible bananas evolved in the Old World by several mechanisms from two wild species—*Musa acuminata* Colla (*M. Cavendishii* Lamb ex Paxt.) and *M. Balbisiana* Colla. *M. acuminata*, which has a primary center of diversity in the Malaysia-Indonesia region, carries the so-called A genome. Plants occur as diploids in both wild (seeded) and parthenocarpic forms. The edible diploid forms (AA) probably arose by the establishment of varying degrees of parthenocarpy and female sterility. The edible (seedless) triploids (AAA) developed through occurrence of triploidy within *M. acuminata* itself. This presumably arose by fusion of a normal haploid male gamete with an egg cell containing a doubled or unreduced chromosome complement. Human selection and clonal (vegetative) multiplication ensured the persistence of the most desirable of these clones. Even so, somatic mutation could have occurred and plants with favored qualities again would have been selected and perpetuated.

M. Balbisiana, of the Indian subcontinent-Malayan region, carries the so-called B genome and occurs only as seeded diploids (BB). When *M. acuminata*, whether diploid (AA) or triploid (AAA), came into contact with *M. Balbisiana* there arose various new forms with the hybrid genomic constitution. The edible diploid forms of these chance hybridizations are seen as originating through the out-crossing of edible

diploids of *M. acuminata* to seeded forms of *M. acuminata* and *M. Balbisiana*, followed by human selection among the edible (parthenocarpic) progeny of these hybridizations. The whole new range of edible triploids arose by haploid pollination of diploid egg cells, e.g., egg cells AA x male gamete B = AAB; egg cells AB x male gamete A = AAB; egg cells AB x male gamete B = ABB. (No edible diploid or triploid *M. Balbisiana* are known.)

Tetraploidy has also been instrumental in the evolution of edible forms of banana. This arose via polyploidy and hybridity. There are some natural edible tetraploids: AAAB, AABB, and ABBB (cf. Richardson et al., 1965); most have been produced artificially and by experimentation with colchicine treatment or pollination of triploids. Again, and as in the case of edible AA or AAA forms, desirable somatic mutations would have been selected and clonally maintained. Simmonds and Shepherd (1956) have pointed out that it is impossible to designate the precise place of origin of these "hybrid" edible bananas but they provide evidence that India was probably the main site of hybridization between *M. acuminata* and *M. Balbisiana*. Accordingly, the Malayan region is generally accepted as the primary center of origin and most of the diversity there is of *M. acuminata* origin. India is the secondary center and the majority of diversity involves the 'A' and 'B' hybrids.

There are, of course, hundreds of clones grown throughout the world. To be taxonomically accurate, the AA and AAA (and even the artificially produced AAAA) should be designated *M. acuminata* Colla. (These have been variously named *M. Cavendishii*, *M. chinensis*, *M. nana*, *M. zebriana*). The edible (seedless) hybrid forms (*M. acuminata* x *M. Balbisiana*) should be designated *Musa* x *paradisica* L. (*M. x sapientum* L.). (In the Genetics and Improvement section of this chapter, the "shorthand" system of designating clones is mentioned in greater detail). It is interesting to note that the 'B' genomes confer some degree of drought and disease tolerance to clones of hybrid origin. Also, increased starchiness and unpalatability of the fruit when raw is associated with the 'B' genome or *Balbisiana* hybridity, but is not an invariable consequence of it. In some parts of the world these starchy cooking bananas are called plantains. All *M. acuminata* types and some hybrid clones have more or less sweet fruits and hence may be eaten uncooked. These are the so-called dessert bananas.

In its diffusion path westwards from Southeast Asia, bananas seem to have gone to Madagascar and from there to the African mainland. The Arab trade routes to the interior facilitated its movement throughout central Africa and ultimately to the west coast. From the Guinea coast, bananas were transported by the Portuguese to the Canary Islands, and from there to Santo Domingo (modern Dominican Republic) in 1516. While it is impossible to say precisely which bananas were introduced, it seems fairly certain that one of them was a cooking plantain. The so-called Canary banana (variously known as Chinese, Dwarf Chinese, Dwarf Cavendish, [Dwarf] Governor), originally from Cochin China (today known as South Vietnam), was not introduced to the Western Hemisphere until the 1820s. Dwarf Cavendish, one of a complex series of bud mutations called the "Cavendish group," is one of the most important and widely cultivated banana varieties in the world.

As one can appreciate, the nomenclatural history at the varietal level is extensive and somewhat confusing (cf., e.g., Simmonds, 1954a,b, 1966; DeLanghe, 1961).

It is impossible in this brief overview to do justice to the historical facts and readers are urged to supplement this necessarily superficial treatment by referring to Kervegant (1935), Reynolds (1951), and Simmonds (1962, 1966, 1976, 1979).

ECONOMIC IMPORTANCE

The importance of dessert and cooking bananas to tropical economies can hardly be exaggerated (Champion, 1963; Haarer, 1964; Simmonds, 1966; Pursglove, 1972). Dessert bananas frequently play a major role as a cash export and are inevitably a complementary food in local diets. Cooking bananas, sometimes called plantains, very often are a staple food and comprise a major part of the caloric intake of large numbers of people in the Caribbean, Central and South America, south central and Southeast Asia, and the tropical West, central, and East Africa (Baker and Simmonds, 1951, 1952; Massal and Barrau, 1956; Anonymous, 1962; Mukasa et al., 1970). Indeed, cooking bananas are increasingly being imported into large urban areas in temperate parts of the world with large ethnic populations who both appreciate and know how to prepare them. The food value of bananas—which has been equated to that of the potato except bananas are usually slightly lower in protein—has been appreciated for a very long time (Myers and Rose, 1917) and continuous efforts are being made to broaden and extend the form in which bananas are marketed. Notable in this regard are specialty items such as banana chips (Palmer, 1979). Some speculate that bananas will play an even greater role—at least where they are grown—as a source of carbohydrate for the production of alcohol (e.g., for the use as a gasoline extender as in gasohol). Needless to say, their importance in the manufacture of specialty beers and spirits (Masefield, 1938)—for instance Ugandan and Sudanese waragi—will be sustained and one occasionally encounters these too in Western markets.

Completely aside from its value as an economic plant, banana has special interest for its morphology, anatomy (see, for example, Barker and Steward, 1962a,b; Fahn, 1953; Fisher, 1978; Mohan Ram et al., 1962; Riopel and Steeves, 1964), physiology (Tai, 1977), and biochemistry (see Steward et al., 1960a,b; Palmer, 1971). From the perspective of growth and development, the presumed involvement of hormones in the flowering process and the subsequent tremendous increase in the pulp tissue of the fruit in parthenocarpic forms are still largely unexplored and challenging problems (see Simmonds, 1953; Steward and Simmonds, 1954).

GENETICS AND IMPROVEMENT

Naturally evolved seedless bananas are perhaps the most conspicuously sterile of all cultivated fruits. Dessert bananas seem to have

derived from *M. acuminata* as edible diploids or triploids (Simmonds, 1962). The artificially produced edible bananas are derived from diploid ($2n = 22$) species *M. acuminata* and/or *M. Balbisiana*. Clones grown on a large scale include diploids, e.g., Pisang Lilan, Bande, Paka, and Ney Poovan; triploids, e.g., Valery, Grande Naine, Lacatan, and Robusta; and tetraploids. The latter are various hybrids predominantly bred by the Imperial College of Tropical Agriculture in Trinidad; the Banana Scheme of the Banana Board, Jamaica (cf. Purseglove, 1972); and more recently by the commercial dessert banana exporters (Purseglove, 1972; Menendez and Shepherd, 1974; Simmonds, 1978). Vegetatively propagated parthenocarpic triploids outnumber the others by far in terms of commercial cultivation and provide all export bananas and plantains.

Since the cultivated bananas are sterile hybrid forms, they cannot be given precise names and until relatively recently the nomenclature has been at best very cumbersome and confusing (cf. Cheesman, 1947, 1948; Moore, 1957, 1958; De Langhe, 1961). This situation was eased when Simmonds and Shepherd (1956) suggested use of the term genome or haploid chromosome set as a key to classification. Using some characteristics these authors categorized the cultivars in terms of relative similarity of the ancestral seeded species. Although genomic designations are known to be oversimplifications, most agree that they serve a very useful purpose in interpreting the origins and patterns of polyploidy. The genome with 11 chromosomes from *M. acuminata* is designated 'A.' The genome with 11 chromosomes from *M. Balbisiana* is designated 'B.' In this scheme, a triploid cultivar or clone of *M. acuminata* such as Valery, Grande Naine, Lacatan, or Gros Michel would be designated *Musa* (AAA group) Valery, Grande Naine, Lacatan, or Gros Michel, or for convenience and brevity AAA Valery, Grande Naine, etc. A triploid cooking plantain, which would frequently be a hybrid between *M. acuminata* and *M. Balbisiana* would be designated, for instance, AAB Laknau, Harton, Domenico, etc., or ABB Chato, Pelipita, Saba, etc. (Simmonds and Shepherd, 1956).

Selection by man for fruit has resulted in the most widely used edible cultivars. As a result of this selection, the ideal dessert fruit, especially in Western trade, is large, long, slender, and seedless, ripens to deep yellow, and is borne in bunches comprised of compact hands on a stalk. Ability to resist damage in transit and to ripen slowly and uniformly over a longish period of time have also played a major role in determining marketability, but by far the most important factors in edible banana production are, of course, parthenocarpy and sterility. A stimulus (or stimuli) to growth of the fruit pulp results in parthenocarpy. Simmonds (1953) and Steward and Simmonds (1954) suggested that auxins and cytokinins or cytokininlike substances are involved. It has been known for a very long time that pollination is not required for production of the edible banana fruit (d'Angremont, 1912). Sterility is the result of a complex of factors, the most important of which is triploidy and attendant meiotic anomalies. This results in seedlessness for, as mentioned above, edible diploids exist. Parenthetically, parthenocarpy apparently does not occur in *M. Balbisiana* since there are no edible (seedless) BB or BBB types (Dodds and Simmonds, 1948). It seems clear, therefore, that parthenocarpy and sterility have arisen via

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gene mutations in fertile diploids; selection and subsequent vegetative propagation ensured their success. A number of structural changes have accumulated in the chromosomal mechanism and this has further exaggerated the sterility (Simmonds, 1962).

De Langhe (1969) has presented at length both the possibilities and difficulties for improvement of banana. He emphasizes that "When all is said and done, the improvement of bananas amounts to a continual fight against fertility, a property, which is, however, the essential instrument of the improvement itself." Menendez (1973a) has provided a summary statement of the activities of the Jamaica Banana Board and again emphasizes the great difficulties. More recently, Rowe (1981, 1984) has provided a general summary of the Banana Breeding Program of the United Fruit Company in La Lima, Honduras. One can rest assured, moreover, that some breeding efforts are being made by a number of the major banana exporting countries but one cannot expect specific details to be generally available.

Because vegetative selection has, until recently, been said to give minimal improvement of dessert bananas, crosses using pollen from male-fertile (AA) cultivars of *M. acuminata* and flowers of male-sterile triploid (AAA) cultivars have been the prime strategy of breeders (Dodds, 1947; Menendez and Shepherd, 1975). Tetraploid progeny, resulting from the fusion of the triploid egg cell of the female and the haploid (A) gamete of the male parent, are produced in very small numbers because of the miniscule number of seeds produced. This, of course, restricts screening trials (Shepherd, 1960). Most breeding efforts have been directed towards the improvement of dessert bananas, the greatest consideration being fruit quality, dwarfness, and disease resistance. Resistance to important diseases such as Fusarium wilt caused by *F. oxysporum* f. sp. *cubense*, Sigatoka leaf spot caused by *Mycosphaerella musicola* (a more severe strain of *M. musicola* has not yet been found in the Western Hemisphere but has been known in the Pacific for many years—cf. Rowe, 1981), Black Sigatoka caused by *Mycosphaerella fijiensis* var. *difformis*, and resistance to the burrowing nematode *Radopholus similis* have been by far the main recent breeding objectives and this, in turn, has necessitated a continued search for germplasm and an interest in the origin of various cultivars and clones. There has been, and still is, considerable interest in assembling germplasm collections for conservation and breeding purposes. The great danger of the monoclonal culture of a disease-susceptible cultivar such as the tall-growing Gros Michel was made very clear in the middle part of this century with the near-decimation of dessert banana plantations in this hemisphere by the Panama or Fusarium wilt disease. Although use of the more dwarf, Fusarium wilt disease-resistant Cavendish AAA clones like Valery and Grande Naine has enabled the industry to survive and flourish, there are apparently some signs, especially in Taiwan, that the Fusarium pathogen can mutate and successfully attack the supposedly resistant Cavendish clones (cf. Rowe, 1981, and refs. there cited). All this emphasizes that efforts still must be made to not only expand the disease-resistant germplasm base but to integrate it into a product that combines all the desirable qualities. This is all the more urgent for it is significant that not a single new culti-

var has been developed for commercial export use in the past 60 or so years (Rowe, 1981, 1984).

Modest programs have been initiated to explore the use of induced mutation breeding (Menendez, 1973a). As a purely clonal crop plant, it seems that bananas are admirably suited to this approach. It is quite clear that the effects of somatic mutation or bud sporting can be significant, since from a historical perspective in Jamaica alone, some six mutants of the once highly prized Gros Michel were detected in a 100-year period and it is surmised that many more have gone undetected (cf. Baker and Simmonds, 1951, 1952). Shepherd (1957) points out that somatic mutation has contributed much to the banana cultivars (clones) in East Africa. Champion (1963), De Langhe (1969), Broertjes and van Harten (1978), de Guzman et al. (1976), and Menendez and Loor (1979) all have emphasized that improvement by mutations induced by chemical mutagens or irradiation has great potential which needs further investigations. Since the theoretical and practical advantages afforded by aseptic culture techniques are particularly relevant here, discussion will be deferred to the next section.

REVIEW OF THE LITERATURE

Embryo Culture

The erratic and generally low germination levels of seeds from many *Musa* clones has rendered use of embryo culture very valuable. Thus this in vitro technique has been in use for a long time (Cox et al., 1960). Because the technique has been described in considerable detail, no attempt will be made to belabor the specifics. A modified Knudson's medium or Randolph and Cox (1943) medium containing 0.12 M sucrose (but without growth regulators) solidified with agar (0.5-0.7% w/v) can be used to rear young plantlets from the tiny embryos excised from banana seeds until they are large enough (that is, several cm tall) to be placed in the soil. The method of Cox et al. (1960) as published applies to *M. Balbisiana* and no references that we are aware of specifically deal with the growing of embryos of other species, but it is clear that this is more or less readily carried out (cf. Rowe and Richardson, 1975).

Excised Shoot Tip Culture

Conventional agricultural practices rely on planting of pieces of corm or rhizome which include at least one bud, small suckers which have only recently appeared above ground level (so-called peepers), sword suckers formed from buds low on the corm, large suckers which have reached the broad-leaved stage (so-called maiden suckers), portions of large corms, etc., etc. (Navarre, 1957; Haarer, 1964; Hamilton, 1965; Turner, 1968; Acland, 1971; Pursglove, 1972). In 1959 a system of maximum multiplication of the banana plant was described (Barker, 1959). The method capitalized upon the presence of "lateral" buds—

actually so-called leaf-opposed buds (Fisher, 1978)—that never achieve any major degree of development under field conditions. This morphological feature of the banana plant attracted Barker and he devised a means of forcing the buds, both those laterally exposed and those that never would be exposed. This involved repeated stripping and removal of the outer leaf sheaths to expose adventitious buds. (It is interesting to note that in the "ensat," sometimes called the Ethiopian banana but more frequently called the False Banana, *Ensete ventricosum* [Welw.] E.E. Cheesm., native farmers have learned to force buds by slicing corms into pieces or removing the central part of the pseudostem from the corm and burying them. New suckers are produced after several weeks. This remarkable practice emphasizes that even in a plant such as *Ensete* where suckering does not take place under field conditions, the potential to release dormant buds may be realized provided the right technique is used—cf. Taye Bezuneh and Asrat Felleke, 1966; Westphal, 1975; Duthie, 1977.)

Investigations have shown that, along the lines shown by Barker (1959) but including refinements made possible by aseptic culture methods and the use of exogenously added growth regulators, excised shoot tips of quite a few clones of dessert bananas are capable of yielding entire plants. Berg and Bustamante (1974) were the first to show that cucumber mosaic virus-free plants of the economically supreme Cavendish group AAA can be obtained from meristems from lateral buds of virally-infected plants by a combination of heat treatment and aseptic culture. In the technique as they describe it, apparently only one plant was obtained per excised shoot apex. The method involved use of Knudson's medium (1946) with the trace elements of Berthelot (1934), thiamine HCl, supplemented with CW 10% v/v and CH. Although the development of roots from such excised shoot tips was rather slow (even after 3 months they had not formed roots), additional work soon demonstrated that roots could be induced to form promptly enough (2 months) with the addition of 5.4 μ M NAA to render the technique commercially useful. Studies from the Republic of China using a combination of semisolid media and liquid media were published around the same time (Ma and Shii, 1972, 1974). For a number of years and until recently, the late Dr. Emerita de Guzman and her colleagues at the University of the Philippines at Los Baños had been making considerable progress in assessing the applicability of the excised shoot tip culture technique to a wide range of clones available to them through the extensive *Musa* germplasm bank at the University of the Philippines at Los Baños. Special attention was given to abaca, *M. textilis*, the source of Manila hemp (de Guzman et al., 1976).

All this work has provided the necessary underpinning for the now extensive use of the shoot tip culture technique (Figs. 1 and 2). One of the major features of the technique as it is now used is that multiplication can be induced by releasing dormant buds at the leaf bases. Subculturing can be carried out from the proliferating mass of shoots which result. Protocormlike bodies can also form at a shoot base. Multiple shoots that originate from these can, in turn, be separated by cutting to produce individual plantlets (Figs. 2c and d and Tables 1-3).



Figure 1. Stepwise sequence of the clonal micropropagation of banana via shoot tip culture. (a) Young plant (1.7 m tall) from greenhouse

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with soil and roots removed to expose corm with two suckers. (b) Corm with side suckers and outer leaf bases of pseudostem removed. (c) Corm trimmed so as to expose the growing point. When explanted this would comprise the apical meristem with one or two leaf primordia. In cases where the material has been demonstrated to be virus-free, special precautions need not be taken to go down to the dome itself. In cases where virus-free plants are to be obtained, the heat therapy treatments according to Berg and Bustamante (1974) should be followed. (d) Cultured shoot split longitudinally through the apex. (e) Same shoot halves, 4 days later. (f) Shoot halves with outer leaves and blackened shoot bases trimmed. Note emergence of side shoots.

Functional roots can be initiated within a few days by using a combination of procedures. (We have observed spontaneous root formation on plants as small as 0.6 cm.) However, root induction can be achieved with far more control and predictability by using low levels of IBA (on the order of 0.01-1.23 μM). In addition, the rooting process can be greatly accelerated by the addition of activated charcoal (0.25% w/v) to a basal medium. Even very tiny shoots (smaller than 1 cm) will produce roots within 4-5 days (Fig. 2e). If an excised shoot tip is placed on a charcoal-containing basal medium, it will begin to produce roots shortly after a recognizable (to the naked eye) shoot has organized. Young plantlets placed on such a medium promptly produce whitish and cream-colored roots (Fig. 2e), and from these propagules plantlets can be established in the greenhouse or in growth chambers within 1 month provided precautions are taken to prevent drying out. In our hands and in the temperate climate of Long Island, New York, a "propagation bed" supplied with an automatic misting system (Mist-A-Matic) in the greenhouse has been very satisfactory.

Callus and Suspension Cultures from AAA *Musa* Clones

The earliest sustained efforts to establish tissue and cell suspension cultures of dessert banana were made in the late 1950s and in subsequent years at Cornell University (Mohan Ram and Steward, 1964). A few isolated attempts have been published since but they have not been impressive, and subculture of callus has not been reported in any papers that we are aware of (cf. Tongdee and Boon-Long, 1973; de Guzman, 1975). Since little of great insight has been published more recently, discussion of callus, suspension, and protoplast techniques will be deferred until later in this chapter.

Key Variables and Factors for Success

Not surprisingly, information is not available as to the full range of *Musa* clones, cultivars, or species that can be rendered pathogen-free or multiplied through the use of shoot tip culture. Since it is very likely that genomic differences in terms of sucker production (so-called stooling properties) may be expressed faithfully, exaggerated, or perhaps

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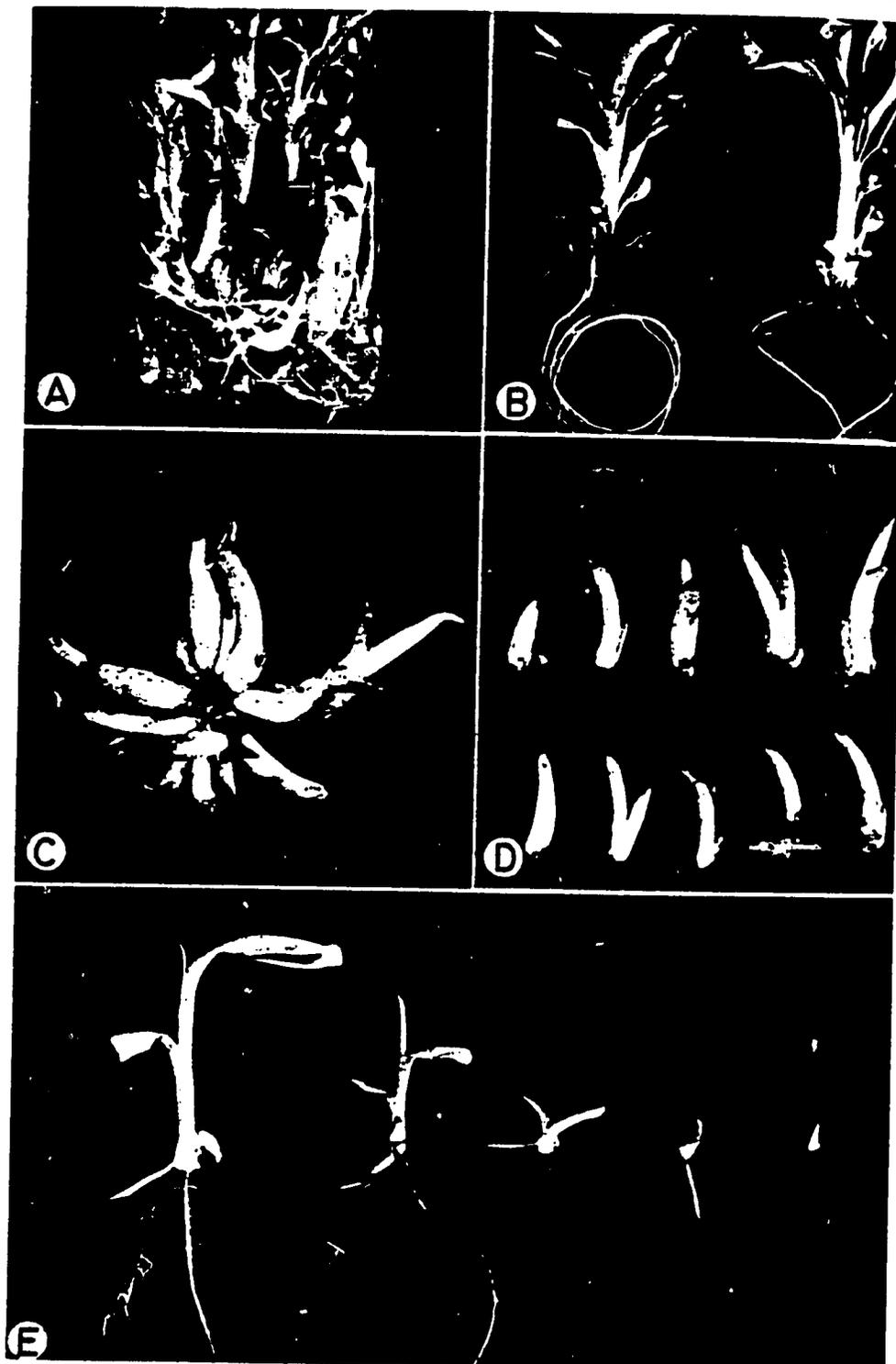


Figure 2. (a) Banana plantlets in culture jar (10 oz) several weeks after subculture. (b) Individual plants removed from the jar. Note

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substantial root development. (c) Close-up showing multiple shoots formed on explant similar to that in Fig. 1f. This emphasizes the origin of shoots from otherwise dormant buds. (d) Individual shoots from the growth form at Fig. 2c separated by scalpel incisions. (e) Rooted banana plantlets. Note that all except the very smallest shoot tip explant has produced roots. Even the smallest can, however, be induced to produce roots (see text for details).

even overcome under conditions of aseptic culture, it will be very useful to have this information. No attempts that we are aware of have been made to ascertain why some produce more side branches than others. Since the plantlets produced in culture are very small (often times as small as a few cm), this could provide a very elegant assay system or means of exposing large numbers of growing points to test conditions. It is well known, of course that *M. Balbisiana* has very variable stooling properties but generally has a much lower tendency to produce suckers. *M. acuminata* subspecies show considerable variation, e.g., *M. acuminata* subsp. *malaccensis* and *siamea* stool quickly and *M. acuminata* subsp. *burmannica* and *banksii* stool slowly (cf. Anonymous, 1962; De Langhe, 1969). Improved stooling properties have been indicated as one of the major breeding aims in cooking bananas of special importance in Zaire (De Langhe, 1969, p. 66). Thus, even in a vegetatively propagated plant like banana one may be faced with the problem of an erratic, irregular or slow stooling response. Trials with cooking bananas (e.g., the clones Pelipita and Saba) suggest that the shoot tip culture methods are as successful as with dessert bananas but the multiplication rates are slower (see also Vessey and Rivera, 1981).

The shoot tip multiplication method obviously has great potential for producing specific pathogen-free planting materials in quantity, and provided precautions are taken, diseases could presumably be eliminated or at least reduced from nursery stock. While ensuring potential reinfection in the field would not be eliminated, use of the method could play a hygienic role in minimizing problems. This would apply especially to presumed virus-caused diseases such as Bunchy Top; leaf mosaic or infectious chlorosis; fungal pathogens such as the Panama Disease, Sigotoka leaf spot, Black Sigotoka, and Black leaf streak; and bacterial diseases such as Moko or bacterial wilt caused by *Pseudomonas solanacearum* (cf. Stover, 1972; Wardlaw, 1972).

From a commercial perspective, the need for rapid clonal multiplication in terms of introducing new clones seems, for the immediate future, to be of limited use because cultivars change exceedingly slowly and new industries are relatively scarce. Most helpful, however, would be the adaptation of the meristem or shoot tip culture technique to germplasm conservation or storage (cf. Withers, 1980, 1981). Ideally, one would like to see the excised shoot tips of as much and as diverse a range of germplasm as is available maintained in aseptic culture under conditions of media composition and/or environment that foster multiplication or branching at the absolute minimal level. Such a system would facilitate storage with a modest input of labor and upkeep. When needed, a culture would be removed from the relatively quiescent state and multiplied as needed by use of the appropriate stimulatory sequence of media or manipulation.

Table 1. Protocol for Banana Shoot Apex Isolation

-
1. Begin with a banana plant or sucker.
 2. Cut off leaves and pseudostem about 30 cm above soil level.
 3. Remove all soil and cut off roots to expose the corm.
 4. Peel off outer sheathing leaf bases of the pseudostem one at a time until they become too small to remove carefully by hand.
 5. Remove the last remaining leaves using a dissecting microscope and a scalpel.
 6. Excise shoot apex with a scalpel.
 7. Sterilize in 1% commercial bleach (0.0525% NaOCl) and Tween 20 for 5 min, swirling occasionally.
 8. Wash 4 times with sterile distilled water.
 9. Place in culture medium.^a
 - Liquid—20 ml in 50-ml Erlenmeyer flask
 - Solid—0.7% agar in snap-lid petri dishes or screw-cap jars
 10. After 3 weeks, transfer to culture medium minus coconut water, solidified with 0.7% agar in screw-cap jars.
-

^aCulture medium:

MS mineral salts	BAP 22.0 μ M	Inositol 5.55 mM
Thiamine HCl 2.97 μ M	Sucrose 0.12 M	CW 15%
pH 5.8	Autoclave	

Table 2. Protocol for Stimulation of Multiple Shoot Formation

-
1. Begin with an aseptically cultured banana shoot or sucker 21 days after explantation.
 2. Make a transverse cut to separate leaves. This yields a section of pseudostem approximately 3 cm long including an intact vegetative bud.
 3. Trim the lower part of explant to remove darkened or necrotic tissue.
 4. Cut pseudostem explant in half longitudinally through the apex.
 5. Transfer halves to culture medium with 0.7% agar in screw-cap jar.
 6. After 4-7 days, remove from culture jar. Trim off outermost leaves and blackened base of explant.
 7. Transfer to fresh medium.
 8. After 2-3 weeks, use a scalpel to cut the multiple shoots that have formed. Transfer shoots thus separated to fresh growth medium.
 9. Maintain the multiple shoot cultures by transferring to fresh culture medium and separating the multiple shoots in the same way every 3-4 weeks.
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Table 3. Protocol for Plantlet Development

1. Separate individual shoots from multiple-shoot cultures with scalpel incisions.
2. Transfer to culture medium supplemented with 0.25% (w/v) charcoal to induce root formation.
3. After 2-3 weeks, transplant into Pro-Mix-Vermiculite (1:1) in small plastic pots and place under mist system.
4. After 7-10 days move to normal greenhouse conditions.

Even now excised axenic shoot tip cultures facilitate germplasm exchange and passage of propagules through plant quarantine (cf. Kahn, 1979). We have used the method with success for banana but precautions must be taken to use a fairly firm agar medium (order of 1%), and a sterile cotton plug should be pushed carefully but firmly against the plantlet or the shoot tip being shipped. In that way any tendency for the plantlet to dislodge in transit is minimized.

Also, large numbers of clonal hybrids that might otherwise be limited to a relatively few specimens can be made available for continued breeding research and field testing. By extension, one can anticipate excising growing points from young seedlings produced through hybridization and inducing them to proliferate and multiply freely, saving much valuable time.

Whatever the rationale, justification, or use, the method must be profitable, for several laboratories are engaged in commercial production for export.

PROTOCOLS

Before presenting a precise protocol for isolation of shoot tips and their subsequent maintenance and growth in culture, it may be useful to restate that different investigators will, of course, utilize different procedures to achieve the same end. Some have recommended that no special effort be made to surface-sterilize the shoot tip since, by virtue of its special morphology, it will generally be free of surface contaminants and pathogens (Berg and Bustamante, 1974). Since the procedure given here is adaptable to obtaining a meristem or a growing point with one or two primordial leaves, we use a surface-sterilizing agent. In the protocol below we recommend the use of sodium hypochlorite so as to minimize the level of contamination. Far too much effort goes into the whole procedure to risk loss of valuable material and we urge that no short-cuts be taken without first establishing the sagacity of the move.

Similarly, some investigators recommend use of media with different components from the one we have outlined. For instance, de Guzman (1975 and personal communication) frequently used Nitsch and Nitsch

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vitamins (1969), citric acid, KIN (in addition to CW), and dextrose (instead of sucrose) as supplements to a basal medium. We examined this in some detail using the same cultivar, Philippine Lacatan, and have concluded that for our purposes the supplementation with KIN or citric acid is neither necessary nor helpful. Similarly, the complex vitamin mixture can be replaced with thiamine HCl (1 mg/l) alone and sucrose can be substituted for dextrose. The CW we use is not essential; it does help a bit. Therefore, investigators are urged to consider the protocol below as merely a guideline protocol and not a problem-free and certain method for all clones.

Shoot Apex Isolation (see Table 1)

1. Shoot apices are isolated from small plants or suckers. The leaves and upper portion of the pseudostem are removed so that a 30-cm stem piece remains. All roots are cut off at the corm and the soil is washed off (Fig. 1a). Any side suckers found on the corm are removed.
2. The outer leaf sheaths of the pseudostem are peeled off one at a time. This procedure is used until the inner leaf sheaths measure approximately 1 cm across at their bases and are 2 cm tall (Fig. 1b). At this point, they become too small to remove by hand.
3. Using a dissecting microscope and a scalpel, the remaining outer leaf bases are carefully removed until only one or two young leaf primordia remain (Fig. 1c).
4. The shoot apex is excised by making four incisions with a scalpel into the corm beneath the apex.
5. The excised apex is placed in 50 ml of a 1% Clorox solution with two drops of Tween 20 made in a sterile 125-ml Erlenmeyer flask. The apex is allowed to soak for 5 min and the solution is swirled occasionally.
6. Working under aseptic conditions, the Clorox solution is decanted and the apex is rinsed four times with sterile distilled water. When the fourth rinse water is decanted, it is poured through a sterile 864 μm (#20) sieve to catch the apex.
7. The sterilized apex is transferred from the sieve to the culture medium using sterile forceps. The culture medium used consists of MS mineral salts supplemented with 5.55 μM inositol, 22.0 μM BAP, 2.97 μM thiamine HCl, 0.12 M sucrose, and 15% CW. It is adjusted to pH 5.8 using KOH. This medium is used as a liquid. Twenty ml are placed in a 50-ml Erlenmeyer flask, which is then stoppered with a foam plug and autoclaved. Apex cultures are maintained at 30 C on a 16-hr light schedule. Liquid cultures are kept on a rotary shaker (100 rpm). The medium can also be solidified with 0.7% Difco agar and poured into 50 x 12 mm snap-tight petri dishes (Falcon #1006). The excised apex can be placed gently on top of the agar and maintained in stationary culture under the same conditions as the liquid cultures.
8. Within 10 days, the growing apex starts to turn green. After an additional 11 days, a small green shoot is clearly visible to the naked eye.

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9. When the shoot becomes about 1 cm long and starts to produce new leaves, it is transferred to the same growth medium minus the CW. This is solidified with 0.7% agar. In this laboratory, the young shoots are often grown in 1-ounce "French square" screw-top bottles under the same growth conditions.
10. An excised apex usually grows into a single shoot or plantlet. Roots usually do not appear on these young shoots although excised shoots only 14 days old have in our hands produced roots longer than 2 cm. Excised shoots can also grow into multiple shoot forms. This type of growth can also be induced as described in the next step (Table 2).
11. A large (3 cm tall) single shoot can be forced into producing many smaller shoots simply by cutting it in half longitudinally through the apex. Using a sterile petri dish as a working surface, the procedure is begun by cutting across the pseudostem to remove the leaf blades, thus producing a stem piece about 3 cm long. If the shoot has produced any roots, they are trimmed off. The stem is cut longitudinally through the apex (Fig. 1d). Each half is placed upright in a culture jar containing culture medium solidified with 0.7% agar.
12. After 4-7 days, the 2 shoot halves are removed and the outer leaves and blackened shoot bases are trimmed off (Fig. 1e). New side shoots are clearly visible at this time (Fig. 1f).
13. After 2-3 weeks, the multiple shoots that have formed are separated by a scalpel incision. These new, smaller shoots should continue to multiply.
14. Our cultures are routinely transferred every 3-4 weeks. Multiple shoots are separated into smaller groups of shoots. This separation procedure induces further shoot multiplication. As the shoots become larger, the cultures are moved into larger jars (Figs. 2a and 2b).
15. Roots are routinely induced by transferring single shoots to culture medium supplemented with 0.25% (w/v) charcoal (Fig. 2c and Table 3). The addition of IBA at the level of 0.1 μM enhances rapid root formation but roots still form without it. In the presence of charcoal, white or cream-colored roots can be seen at the shoot base in 4-5 days. Plantlets can be potted in Pro-Mix-Vermiculite in small plastic pots in about 2-3 weeks. They can be moved to normal greenhouse conditions in 7-10 days.

Mutation Breeding and Mutagenesis

Champion (1963), Stotzky et al. (1964), De Langhe (1969), Menendez (1973a), de Guzman (1975), de Guzman et al. (1976), Broertjes and van Harten (1978), and others all have seen and emphasized the benefits that could accrue if the various methodologies for producing dessert banana and plantain mutants could be worked out, applied, and assessed. In most of the above cases, mutagenesis of suckers using γ -irradiation prior to excision and culturing of the growing point has been the point of departure (cf. Broertjes and van Harten, 1978, pp. 256-258), but Menendez (1973b) has also reported use of the chemical

mutagen ethyl methane sulphonate on seeds of *M. acuminata*. The field is only now beginning to realize some of its potential (Menendez and Loor, 1979). De Guzman and her colleagues regularly used about 2.5 Krad to irradiate suckers prior to culture but they encountered much variation in responsiveness depending on the clone (de Guzman, personal communication). Some clones actually grow faster as a result of irradiation and in others the growth is attended by a dramatic slowing down. The gross morphology of the cultures is also affected, those being irradiated tending more towards a compact protocormlike habit. No attempt is made here to outline the procedures since general principles are available (cf. Broertjes and van Harten, 1978).

From our perspective the most challenging and rewarding work in this area is sure to derive from the eventual application of mutation breeding techniques to totipotent cell suspensions or protoplasts but this will not be easy to achieve (Krikorian and Cronauer, 1983). Even so, work in this laboratory with a recalcitrant perennial monocotyledon, the daylily (*Hemerocallis*), has shown that perseverance can lead to significant progress (cf. Krikorian and Kann, 1979, 1980, 1981; Krikorian et al., 1981; Fitter and Krikorian, 1981).

The production from banana of true callus and/or rapidly growing morphogenetically competent cell suspensions will be a major step towards generating improvements in banana as a crop. These techniques will greatly enhance the chances of selecting potentially useful mutations if plantlets can be caused to arise from single cells. Consequently, much interest has been expressed in the initiation of totipotent callus cultures and subsequent suspension cultures that have a capacity for production of plantlets either by initiation of adventitious shoot and root growing points on a cell-derived callus, or by the formation of adventive or somatic embryos (Steward et al., 1975; Steward and Krikorian, 1979; Krikorian, 1982; Krikorian and Cronauer, 1983).

An examination of the literature demonstrates how recalcitrant the banana has been in terms of the induction of totipotent cell suspensions or callus tissue. Mohan Ram and Steward (1964) used a variety of auxins to induce callus formation on sections of mature and immature fruits. The callus tissues and the cell suspensions they obtained when placed in liquid medium were slow-growing and showed no signs of organized development. Although later workers (Tongdee and Boon-Long, 1973; de Guzman, 1975) also reported the production of callus from banana fruits of several clones, they were equally unsuccessful in stimulating any organogenesis. Attempts in this laboratory to evoke callus which can be subcultured from immature and mature but pre-climacteric fruit sections have not been encouraging. Although proliferating tissue masses can be obtained, "growth" is predominantly by cell enlargement, and the potential for any morphogenesis or organogenesis from such fruit-derived tissues, given the state of the art, seems extremely limited at present. A major problem to be overcome involves the substantial tendency of fruit tissues to blacken due to oxidation of polyphenols. In our view it is much more likely that cultures derived from other somatic tissues will have a greater potential for producing totipotent cell suspensions and work is progressing in that direction.

Cell suspensions might also be obtained through protoplast culture. Such cultures could offer yet another means of inducing or even introducing variation into banana. We have been able to prepare protoplasts reproducibly from various parts of dessert bananas and are working towards establishing cell suspensions from these preparations (Cronauer and Krikorian, unpublished). Since no plants have yet been recovered from protoplasts, we hesitate to provide details here.

FUTURE PROSPECTS

The distinctive evolutionary origin of edible bananas and plantains and their high potential for clonal multiplication provides many opportunities to the tissue culture worker. Wild (seeded) and edible (seedless) *M. acuminata* exist as normal diploids as well as triploids. Only seeded diploid types of *M. Balbisia* occur but even some of these are economically valuable. Parthenocarpy and sterility within triploids; outcrossing of edible diploids with seeded forms of *M. acuminata* and *M. Balbisia*, followed by human selection; occurrence of triploidy within *M. acuminata*; and triploidy in crosses between *M. acuminata* and *M. Balbisia* (giving rise to two genetically different kinds of triploids), have each played their part in the origin of the many kinds of edible bananas available today. Dwarf, medium, and tall growth habits with varying intergrading forms are known. Variation in habit, fruiting behavior, fruit color, and quality abound. Somatic mutations have further expanded the base of diversification.

Artificial means of inducing tetraploidy and higher levels of polyploidy by use of colchicine emphasized some time ago that man can further intervene in manipulating the genomic composition. (cf. Vakili, 1967). The use of radiation and chemicals for mutation breeding has shown that a real potential exists for each. But as the kinds of techniques described in Volume 1 of this series are developed and perfected for application to *Musa* clones and species, a whole new level of potential will emerge. We have mentioned the use of totipotent cell suspensions for rapid clonal multiplication. Mutagenesis using free morphogenetically competent cells or protoplasts has also been mentioned. To this may be added use of controlled protoplast fusion to achieve a still greater combination of characters hitherto unobtainable (Krikorian and Cronauer, 1983). Androgenesis and pollen culture, if achievable, could play a significant role in all this. In short, the potential is there, one merely has to develop it. Unfortunately banana must be classified as a rather recalcitrant system at this time for all procedures other than embryo culture and shoot tip culture. Whether this state of affairs is a reflection of the lack of research emphasis until rather recently, or the fact that we lack a fundamental scientific base from which to operate, will soon become apparent. This is because several groups are now engaged in basic tissue, cell, and protoplast studies (cf. Withers, 1981).

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Técnicas de cultivo aséptico para el mejoramiento del banano y plátano

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INTRODUCCION

Para los lectores del "Informe Mensual" no es necesario recordar que la Sigatoka negra causada por el hongo *Mycosphaerella fijiensis* var. *difformis*, está ocasionando estragos en varios países productores de banano y plátano.

Por un tiempo se podrá mantener un buen control fitosanitario mediante el uso de agroquímicos, pero estos son tan costosos, que aún los grandes productores no podrán sostener los gastos sin sufrir consecuencias económicas; está fuera de discusión el efecto de la enfermedad en los agricultores en pequeña escala, lo cual puede conducir a un desastre.

Existe una inmediata y urgente necesidad de identificar o producir clones de banano o plátano resistentes o tolerantes a la "Sigatoka negra" (Stover, 1980).

Esto debe hacerse tan rápido como sea posible, pero cualquier programa que pretenda encontrar soluciones al problema de la Sigatoka negra, debería tomar ventaja de esta oportunidad para que, en forma simultánea, se diseñen los procedimientos en el caso de que se presente una nueva enfermedad.

En la actualidad, no se pueden dejar de lado las crisis en políticas de manejo de importantes cultivos y el banano no es la excepción.

El objetivo de producir clones tolerantes o resistentes a Sigatoka negra (o a cualquier otra enfermedad en este cultivo), se realiza por los métodos convencionales de mejoramiento basados en la hibridación, el cual es un proyecto a largo plazo, debido a que la resistencia tendría que ser buscada en fuentes de diploides silvestres.

Es decir, se necesita encontrarlos al inicio de una larga secuencia de mejoramiento, diseñado para hallar un poliploide comercial.

Del cruzamiento que se efectúe usando polen de clones masculinos fértiles y flores de clones mas-

culinos estériles, pero fértiles del lado femenino, se obtendrían al principio clones triploides; pero ello no significa que sea la única estrategia de los fitomejoradores del banano.

La progenie tetraploide, resultado de la fusión de una célula embrionaria triploide femenina y el gameto masculino triploide, se produce en escaso número, debido al minúsculo número de semillas que se originan (Rowe, 1981).

Se requieren cerca de tres años antes de que se pueda ver el resultado de un cruzamiento y se ha estimado que se podrían necesitar 10 años, desde el desarrollo inicial, para obtener un adecuado clon de banano tolerante a la Sigatoka negra.

Además del hecho de que no puede haber garantía de que tal cruzamiento necesariamente conduzca a un producto comercializable, se presenta el problema adicional de que el cruzamiento posiblemente no puede llevar la misma velocidad que el desarrollo de nuevas razas de los patógenos, potencialmente más virulentos.

Los esfuerzos que se hacen para encontrar nuevos clones comestibles, que de algún modo han escapado a su detección, con características deseables (resistencia, etc.), es optimista y tiene sus propios y especiales problemas, incluyendo el mercadeo y la aceptación de los consumidores.

El banano de exportación cultivado para el mercado occidental, debe ajustarse a rigurosos requisitos y no es realista pensar que se puede encontrar un clon para postre que combine un sinnúmero de características excepcionales. Desde el punto de vista de los autores, es mayor la posibilidad de identificar clones de plátano por este medio.

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Asimismo en el sureste asiático, donde se presenta la mayor variabilidad de germoplasma del género *Musa*, se encuentra el problema de la enfermedad viral denominada "cogollo racemoso" o bunchy top (Stover, 1972; Wardlaw, 1972).

Esta es una de las enfermedades virales que, hasta el momento, no se ha presentado en el hemisferio occidental y debería ser esencial asegurar que no se introduzca en forma inadvertida; en la actualidad, es insuficiente la evidencia acerca de si se tiene un método relativamente simple que garantice la eliminación de todos los virus del punto de crecimiento de una planta infectada.

En vista de todo esto, la necesidad de utilizar otros métodos, solos o en combinación con los programas de mejoramiento convencionales para producir bananos y plátanos mejorados, comienza a verse como una posibilidad real.

El uso de técnicas de cultivo aséptico asociado a un programa de mejoramiento, puede mirarse de varias maneras. Estas técnicas pueden proveer un medio para hacer disponible el germoplasma ordinariamente accesible. Lo anterior puede realizarse en virtud de los procedimientos de preservación de embriones exentos de patógenos específicos, facilitando la introducción de clones de diferentes localidades o países.

La multiplicación por medio del cultivo aséptico de especímenes generalmente escasos, es otro camino para incrementar su disponibilidad.

Se tratará más adelante acerca de estos métodos, pero es en el potencial de otras técnicas de cultivo, incluyendo la inducción de variación (que puede ser utilizada en un conjunto de programas de mejoramiento combinado), en el cual se hará énfasis en este documento. De esta forma se presenta algo más elaborado, que un simple panorama de algunas de las estrategias o posibilidades, en las cuales se utilizan las técnicas de cultivo aséptico (Krikorian 1982).

• CULTIVO DE EMBRIONES

Hace bastante tiempo, a principios de 1920, se demostró que algunas veces se puede estimular el crecimiento de ciertos embriones en cultivo aséptico, que de otra manera no se obtendrían o se harían en forma irregular.

Desde ese tiempo, el cultivo de embriones se ha aceptado como un procedimiento rutinario, para

preservar aquellos que normalmente no continuarían su crecimiento hasta convertirse en plántulas.

El cultivo de embriones de semillas de *Musa*, ha sido una valiosa ayuda en los programas de genética y la técnica ha sido muy útil, por ejemplo, en el mejoramiento de diploides resistentes a *R. similis* (Rowe y Richardson 1975, p. 26).

Actualmente la técnica se utiliza con perfección pero es limitada la información publicada al respecto. Se necesita aprender mucho más acerca de este procedimiento.

Una faceta del cultivo de embriones que no ha sido encarada totalmente, involucra la posibilidad de producir plantas triploides (AAA, BBB, ABB o AAB), por cultivo y manipulación del endospermo triploide normal de semillas diploides.

• CULTIVO DE MERISTEMAS APICALES O SHOOT-TIP

El descubrimiento en la década del sesenta, de que cortando los puntos de crecimiento y ápices de orquídeas del género *Cymbidium* cultivadas asépticamente, cuando se cortan apropiadamente, podrían producir protuberancias que se asemejen a protocormos normales, las cuales pueden crecer hasta convertirse en plántulas, proporcionando el impulso más dramático para el ulterior desarrollo de procedimientos para la multiplicación y mantenimiento de plantas en cultivo aséptico.

Estas técnicas se han utilizado en muchas plantas para la obtención, mantenimiento y multiplicación de un gran número de individuos en espacios reducidos.

En algunos casos, se genera una sola planta de un ápice y en otros se puede estimular la formación de ápices múltiples.

Mientras se desarrollan ápices que emergen y proliferan del punto de crecimiento de un explante, pueden mantenerse a una tasa continua por la remoción efectuada mediante cortes y de esta manera se tiene un sistema abierto.

Solamente se mantiene un balance que favorece la formación continua de crecimientos menos diferenciados, que pueden organizarse en cultivos ajustando el medio.

Cuando estos ápices (con o sin raíces), se sacan de la masa proliferante y se transfieren a otro am-

biente o a diferentes medios conductivos, para el ulterior desarrollo radicular, las nuevas proliferaciones los reemplazan (fig. 1a-1d).

Utilizando el sistema de aislar únicamente el ápice, Berg y Bustamante en 1974, demostraron que se pueden obtener plantas del subgrupo Cavendish AAA, libres de virus, a partir de meristemas y yemas laterales de plantas con virosis, mediante una combinación de tratamiento térmico y cultivo aséptico.

Desde ese entonces, los procedimientos de cultivo se han mejorado considerablemente, de tal manera que se pueden producir en un corto lapso, grandes cantidades de ápices y éstos a su vez, pueden ser enraizados en 3 ó 4 días (Krikorian y Cronauer, 1983).

mantener material libre de bacterias y hongos con el propósito de intercambiar, transferir y transportar germoplasma (Stover, 1977).

En el caso de las enfermedades virales se presenta más de un problema.

El virus del mosaico del pepino (CMV) puede ser eliminado, pero no hay certeza de que con otros virus el material esté exento de ellos; por tanto se necesita aún más trabajo de investigación en ésta área.

Se debe realizar un esfuerzo especial para desarrollar un material que sea estrictamente axénico.

Existe una buena posibilidad de que esto podría conducir a obtener plantas más vigorosas y a incrementar las cosechas.

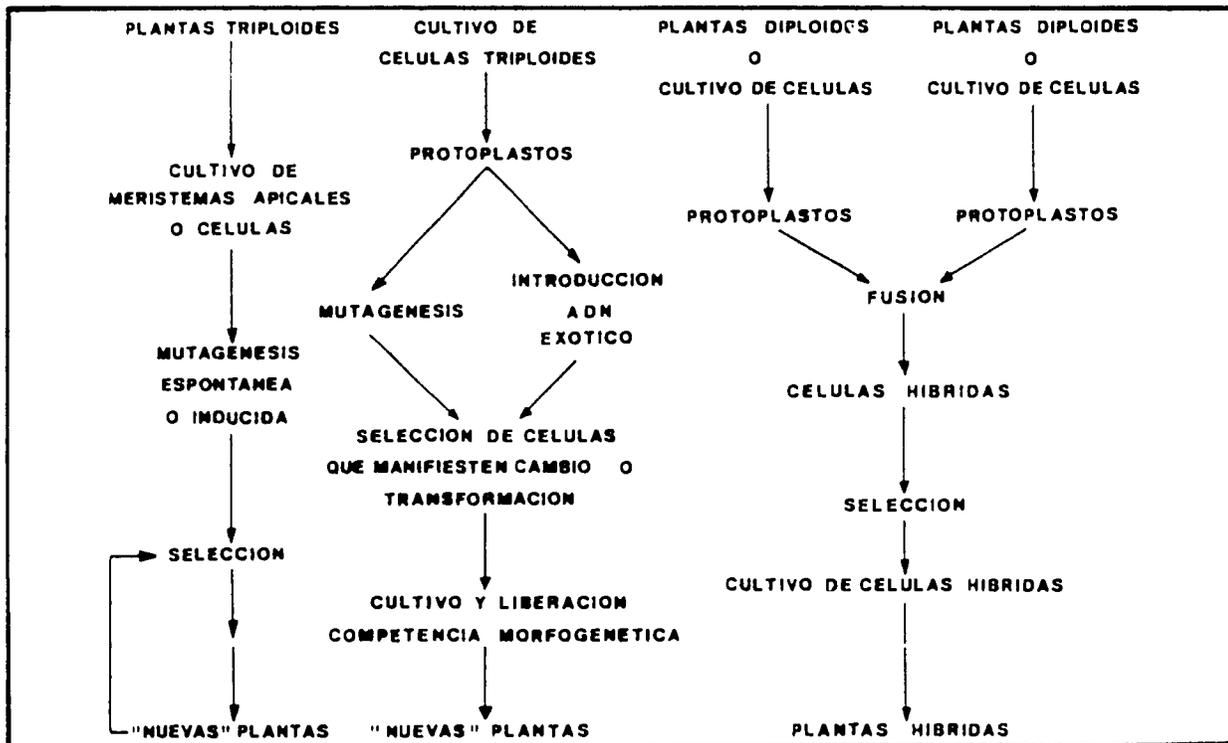


Fig. 2. Modelo para el posible uso de técnicas de cultivos de células, protoplastos y meristemas apicales para producir nuevas plantas de banano o plátano.

Obviamente este método de multiplicación tiene gran potencial para la reproducción de especímenes, a partir de los esfuerzos de los programas de mejoramiento, los cuales no son ordinariamente disponibles en forma rápida y además para la producción en gran escala de material de siembra libre de patógenos.

Puesto que el procedimiento se efectúa en condiciones de asepsia, los ápices pueden ser útiles para

Desde una perspectiva comercial, la necesidad para una rápida multiplicación clonal (en términos de introducir nuevos clones), hasta hace poco no fue vislumbrado como una limitación mayor, puesto que los cultivares de banano para exportación habían cambiado de manera excesivamente lenta y los nuevos trabajos fueron relativamente escasos.

Sin embargo, se palpa la necesidad de suministrar grandes cantidades de material sano para la siembra

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de nuevos clones, con el propósito de reemplazar aquellas plantaciones que por cualquier razón se han deteriorado.

De manera ideal, se desearía tener meristemas de muchos clones y especies provenientes de diversas regiones geográficas conservados en bancos de germoplasma.

Se podría entonces mantener el germoplasma en cultivos asépticos bajo condiciones ambientales o de medios de cultivo que mantengan la multiplicación a un nivel mínimo. En caso de que se necesitara el cultivo, podría removerse de un estado de relativo reposo y se multiplicaría mediante el uso de una secuencia de estimulación apropiada o manipulación del medio. La posibilidad de todo esto, es una necesidad real que debe llevarse a cabo.

Los autores estiman que se tienen numerosas razones para creer que es factible realizarlo.

• MUTACIONES

Cultivo de meristemas apicales

Varios investigadores han hecho énfasis en los beneficios que resultarían de la producción de mutantes de banano y plátano utilizando las técnicas de cultivo asépticos. El poco trabajo que se ha realizado, ha involucrado el uso de rayos gama antes del corte y cultivo del meristema o punto de crecimiento.

Solo hasta ahora comienza a reconocerse el potencial de este campo para el cultivo del banano (Menéndez 1973; Menéndez y Loor 1979).

Dado que son pequeñas las plántulas producidas por medio de multiplicación de meristemas en asepsia, éstas se pueden utilizar convenientemente para la manipulación de mutagénesis.

La producción controlada de variantes no es materia trivial, puesto que las variaciones somáticas han contribuido grandemente a la diversidad del banano.

Se conoce la existencia de mutaciones en el banano, muchas de las cuales se han presentado repetidamente. Algunas de ellas se han tornado importantes desde el punto de vista económico, como es el caso de la producción del clon Highgate, el cual es una variación del "Gros Michel".

En vista de que la base genética para el mejoramiento del banano para exportación es muy limita-

da del lado femenino, (por ejemplo todas las "madres" son "Gros Michel"), entonces la disponibilidad de líneas de germoplasma femenino tiene un gran valor.

Realmente, si se produjesen variantes de "Gros Michel" o "Grand Nain", etc., la labor de los genetistas sería más fácil.

De la misma manera, la producción de tetraploides por endopoliploidización espontánea en cultivos diploides o mediante el uso de colchicina, sería de gran beneficio.

Se sabe que la multiplicación vía meristemas tiene potencial, per se, y bajo condiciones especiales para producir una substancial variabilidad sin el uso de agentes mutagénicos.

Por ahora no se puede estar seguro que esa variabilidad incluiría resistencia a Sigatoka negra, pero ello podría ser mejorado.

• Utilización de células y cultivo de protoplastos

Desde la perspectiva de los autores, el trabajo más desafiante y del cual se obtendrían mayores beneficios, es la seguridad que se obtenga de la eventual aplicación de las técnicas de mejoramiento (por medio de mutaciones) a las células y protoplastos.

Esto se vincula con el uso de células que tienen capacidad para la producción de plántulas a partir de poblaciones de células libres, en contraste con el uso de meristemas organizadas las cuales están compuestas de muchas células. Estas células pueden convertirse en plantas y son llamadas totipotentes.

La probabilidad de seleccionar mutaciones de gran potencial por medio de las técnicas de cultivo de células, sería de gran importancia puesto que se afectarían muchas células simples y cada una de éstas podría, al menos teóricamente, ser capaz de crecer hasta convertirse en una planta.

La inducción de suspensiones de células totipotentes de cualquier planta, se estima generalmente como difícil de ejecutar pero se debe hacer el intento.

Es un hecho conocido de que los cultivos de células pueden crecer de una manera tal como para que se produzca variación y ciertamente en forma

más factible que lo que se obtendría por medio del mejoramiento convencional.

Si a lo anterior se une el uso de agentes mutagénicos, las posibilidades de mejoramiento por medio del cultivo de la suspensión de células, se podrían ulteriormente multiplicar los protoplastos o células libres de su pared celular, mediante el uso de enzimas que degradan la celulosa, lo cual ofrece un medio aún más sofisticado de inducir o aún introducir variación, en una planta que se propaga vegetativamente, como es el caso del banano.

Asimismo se puede vislumbrar, eventualmente, la posibilidad de la fusión protoplásmica entre diploides comestibles para hacer tetraploides.

Superando, por medio de la preparación de protoplastos, la barrera para la fusión celular la cual es inherente a la presencia de una pared celular, la fusión de protoplastos podría conducir a la fusión de los núcleos entre clones no relacionados e inducir a la producción de células reconstituidas, a partir de las cuales las nuevas plantas puedan desarrollarse.

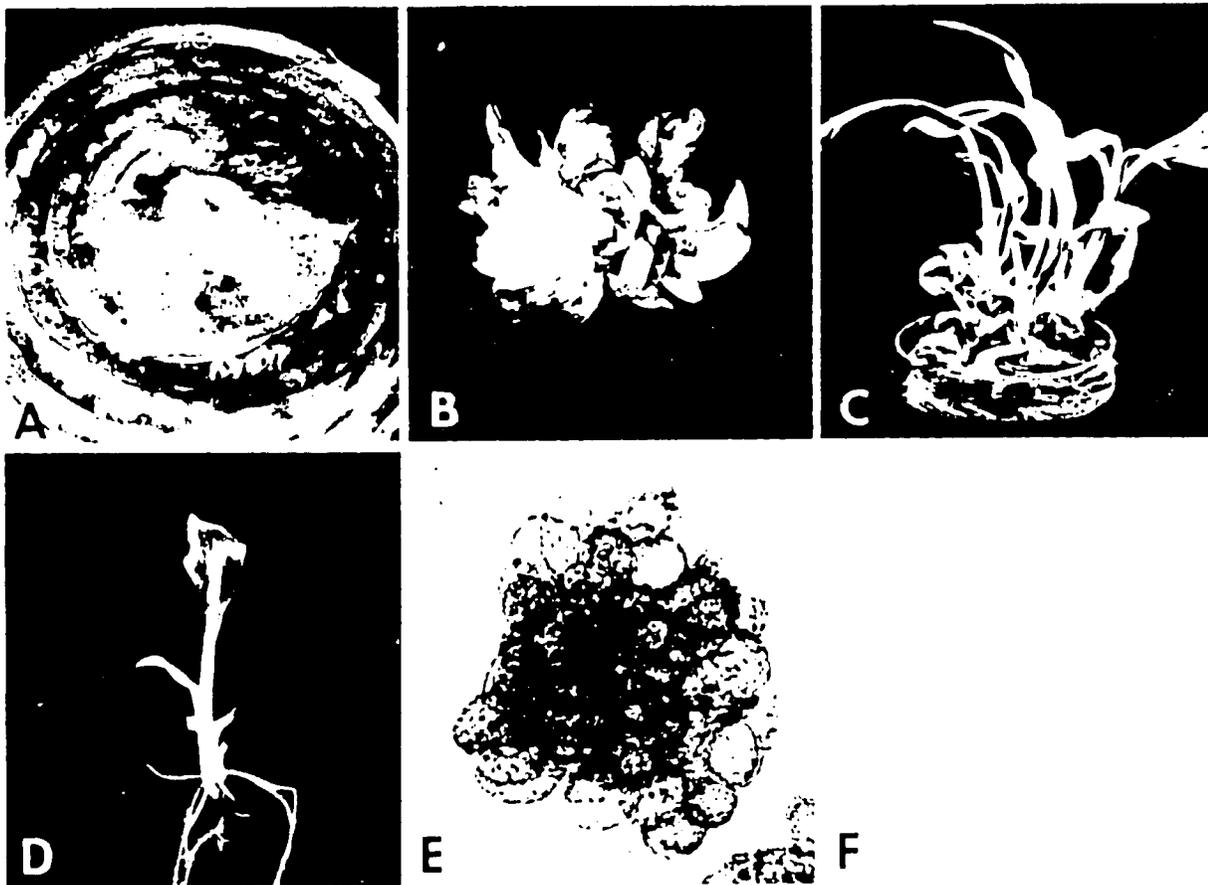


Fig. 4. Fotografías representativas de varios niveles de la técnica de cultivos aplicadas a bananos y platanos. a. exposición del punto de crecimiento antes del corte (1X); b. extremidad del tallo con múltiples yemas alrededor de la periferia (1.5X); c. última etapa de multiplicación de ápices por medio de aislamiento del meristema (0.33X); d. plántula enraizada asepticamente lista para transplantarse (0.55X); e. grupo de células creciendo en suspensión (99X); f. protoplastos preparados por medio de digestión enzimática de la pared celular (980X).

De manera ideal se podrían preparar protoplastos, exponerlos a agentes mutagénicos y entonces cuidarlos hasta que se conviertan en plantas.

Lo anterior difiere del solo cultivo de células, en el cual se puede comenzar en forma directa con células procedentes de la planta adulta sin aislar e inducir células totipotentes en el cultivo.

La figura 2, muestra cómo este proceso puede llevarse a cabo; también describe cómo las células podrían ser modificadas utilizando agentes mutagénicos y técnicas de ingeniería genética, en combinación con el cultivo de células y métodos de utilización de los protoplastos.

Sin embargo, se enfatiza que si bien se anticipa la limitada utilización de éstas ideas y métodos, a

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pesar de las pretensiones que sea una técnica accesible y útil en los métodos rutinarios de investigación, se deben perfeccionar los conocimientos de todas las técnicas del cultivo aséptico relacionados con el género *Musa*.

La atracción del uso de éstas en banano y plátano, es que los métodos ya existen y que se puede llegar lejos para encontrar solución a algunas de las necesidades más inmediatas.

De manera simultánea deben encontrarse las demandas que se requieren para proyectar los fundamentos necesarios a un largo plazo, relacionados con los problemas básicos que involucra una verdadera "ingeniería genética", utilizando todo el conocimiento del genoma *Musa*.

En la Universidad de New York, Stony Brook (Long Island), se está trabajando hacia la determinación de si el cultivo aséptico de meristemas, células o protoplastos, tienen algún potencial para el mejoramiento del banano y plátano.

Si bien no se anotan detalles al respecto, los autores creen que se presenta la coyuntura para hacer esfuerzos concertados sobre este problema, y no esperar que lo realice un grupo en forma aislada.

Se necesitan investigaciones que determinen los parámetros exactos, para el establecimiento y crecimiento de cultivos de tejidos, células y protoplastos "totipotentes"; luego se necesitará definir las condiciones óptimas para poner a prueba las células y protoplastos totipotentes, a los patógenos y agentes mutagénicos.

Se debe prestar especial atención a la restitución de los sistemas, de forma tan confiable como sea posible y llevar a cabo estudios comparativos, en un amplio intervalo, del material clonal o biotipos promisorios y adecuados para tales pruebas.

Un sistema capaz de desarrollar plantas completas a partir de una simple célula o protoplastos, es una característica crucial y esencial de la ingeniería genética.

Sin ésta aptitud, no existe medio confiable de producir o seleccionar nuevo germoplasma totalmente resistente.

El cultivo de meristemas tiene su lugar y es potencialmente muy valioso, pero no puede sustituir los sistemas de cultivo de células y protoplastos ■

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