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International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

# Collaborative Research Programme

# TERMINAL EVALUATION REPORT

**UNIDO contract #** 91/056

**ICGEB ref. #:** CRP/ NIG 90 - 01

**Project initiation:** 1991

**Project termination:** 1994

International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

# Collaborative Research Programme

## TERMINAL EVALUATION REPORT

Part 1

**Title of Project**

THE BIOLOGY OF BANANAS, PLANTAINS. AND OF SIGATOKA IN THE BREEDING FOR RESISTANCE TO THE SIGATOKA LEAF SPOTS

**Keywords:** Sigatoka, Bananas, Tissue Culture, Chemotaxonomy, Electrophoresis, Isozymes, Phyto-toxin, Cell selection.

**UNIDO contract #** 91/056 **ICGEB ref. #: CRP/** NIG - 90 - 01

**Project initiation:** 1991 **Project termination:** 1994

**Principal Investigator's name:** TUNDE FATUNLA

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**Abstract:**

Stomatal inflorescence and fruit type variations are useful taxonomic tools in banana and plantains.

Electrophoretic characterization of crude protein and enzyme extractions (peroxidase, tetrazolium oxidase, and malate dehydragenase) are useful in indexing. It was established that all the three Mycosphaerella species, M.fijiensis var. difformis, M. fijiensis, and M. musicola are present in Nigeria. Pure cultures of each species were produced. It was also demonstrated that the crude filtrates from the mycelium produce necrotic lesions on susceptible plant leaves both in the greenhouse and on leaf discs. The toxins are not enzymes since they were still active after being heated at 120°C for 20 minutes.

Callus tissues were established from resistant and susceptible plantain and banana cultigens. Resistant lines were obtained from susceptible cultigens through the use of increasing concentration of toxin on callus tissue by recurrent (cyclical) selection.

Part 2

## OBJECTIVES/METHODOLOGY
**(proposed at the time of the submission of the research proposal)**

(a) **Objectives**

1. Quantitatives and biochemical characterization of plantain clones in Nigeria.
2. Identification and description of Mycorphaerella species present in Nigeria.
3. Extraction and chemical characterization of toxins produced by the mycosphaerella species.
4. Development of tissue culture technique for banana improvement.
5. Assemblage of information that will be useful in converting susceptible plantain/banana to resistant clones.

(b) **Methodology**

1. Limited Collection of plantain/banana cultivars.
2. Classification of cultivars using morphological traits such as variation in forms (a) inflorescence types (b) fruit types (c) stomatal variation
3. Use of electrophoretic mobility of crude proteins and isozymes to separate clones.
4. Through spore pick technique, pure cultures of Mycosphaerella spp. were obtained and on different growth media (Potato dextrose Agar, PDA), Mycophil agar, v-8 juice agar, malt extract agar.
5. The colony colour, diameter, rate of colony growth, pigment, length of conidia, septation of conidia, size and shape of spores were used to separate the collection into the three different species.
6. Protoplast culture from callus tissue was to be produced.
7. Production of toxin from mycelium
8. Using different plantain and banana cultigens serological studies and toxin characterization, the variability in the pathogen was to be established.
9. The potential of the use of the toxin in early screening and mass screening in cultures will be evaluated.
10. The differential reactions of tissues to toxins of known concentrations by susceptible, tolerant, resistant and immune cultigens will provide information on the utility of the toxins for selection on explants and on calluses.
11. It was left open to use plant transformation or cell selection to develop resistant clones.

## RESULTS
## (compare against the set objectives)

1. Quantitative characterization of plantain clones in Nigeria.

   (i) Stomatal density of mature plantains and bananas ranged from a mean of 4.0 - 10.0 for the adaxial, 28.0 to 36.0 for the abaxial surfaces respectively per microscope field at x400 mag. The mature culligens stomatal mean length, width, pore size and area range from 14.8[1] - 20.3 um, 9.7 - 19.6 um, 2.8 4.6 and Guard cell area 113 - 259 at X400 mag. All the parameters showed significant differences among culligens at 5% level. There were also significant difference between stomatal densities in the adaxial, abaxial surface and among the basal, middle and tip areas of the leaf for each trait measured.
   The result showed that stomatal differences in combination with other morphological traits could be used in ploidy, group and individual culligen identification.

   (ii) Length and diameter of fingers, fruit curvature, shape of fruit, number of fingers/first and second arms, number of arms/bunch varied significantly among cultivars and could be used to characterize culligens.

2. Biochemical Characterization of Culligens.

   The result from leaf protein, tetrazolium oxidase and peroxidase studies demonstrated that the 12 clones which had been hitherto grouped into four using morphological traits were separated into 12 different culligens.
   The peroxidase and malate dehydrogenase isozymes could be used to separate the clones into AAB, ABB and AB genomic groups.
   The potential of the use of similarity index in studying variations in banding patterns was also demonstrated.

3. Identification and description of Mycosphaerella species present in Nigeria. We have established that the three Mycosphaerella species M. fijensis, M.fijensis var. difformis, M. musicola are present in Nigeria. M.fijensis and M. fijensis var. difformis were very similar. However on v.8 juice agar M. fijensis var. difformis developed an earlier and pink colouration than M. fijensis.
   M. musicala grew faster than the other two species on PDA and V-8 juice agar.

4. Extraction and chemical characterization of toxins produced by the Mycosphaerella species. Type of media, PH affected the yield of toxin. It was observed that the toxin was still phytotoxic when the extract was heated at 100°C for 30 minutes. The toxin is therefore not an enzyme and the active substance is heat stable.

5. Development of tissue culture technique for banana improvement
   (i) Establishment of plantain/banana tissue cultures.
   Shoot apices isolated from the culligens were obtained after sterilizing in sodium hypochlorite (2.0%) for 30 minutes. Short tips with two to four leaf primordia and basal rhizome part (2 - 3cm) was further sterilized in a solution of sodium hypochlorite (10.7%) and ascorbic acid (40 mg/l). After rinsing in sterile distilled water, they were aseptically transferred to the growth medium. These eventually produced proliferating tissues. To obtain an optimal proliferating rate, clusters of the white meristems that become visible after removing the leaves were regularly transferred to fresh modified MS medium.
   (ii) Regeneration of plantain and banana
   The shoot meristem tips isolated from the proliferating cultures, corms, suckers and peepers of all the cultivars gave 100% survival in both liquid and solid media.
   The problem of blackening which occurred with the cultivars was overcome by adding ascorbic acid and L-cystein HCL and also by frequent transfer into fresh medium.
   Low BAP concentration resulted in single shoot yields. Either IBA or NAA promoted root initiation. Negative geotropic root growth was arrested by putting rooted plantlets at the lower section of the incubator and by covering the lower section of the tube with aluminum foil
   Plantlets which had rigorous shoots and roots were transferred to sterile soil.

6. Assemblage of information that will be useful in converting susceptible plantain/banana to resistant clones.
   (i) Callus production and culture.
   Callus cultures were initiated from shoot tips (5.0 mm) from proliferating cultures and placed on either modified M & S, or Gramborg medium.
   Callus tissues were best produced in darkness for all the genotypes studied. Callus initiation and quality was influenced by the concentration of Decamba in the culture media. When 2,4 -D and BAP was applied, Callus was produced by all the cultivars.
   There were genotypic differences in response to auxin and cytokinin treatment for both callus proliferation and plantlet regeneration from callus.
   (ii) Determination of Sensitivity of the Cultures to toxin.
   Callus cultures were grown on media containing a wide range of toxin concentration(10 - 100 ug/l). Cyclical/recurrent selection technique was used to select resistant callus tissues/cells. Surviving calluses and which were pre-embryonic were transferred to modified M & S medium and regenerated to plantlets.
   (iii) Response of Callus tissues to the toxin
   Susceptible cultivars could not survive on medium with toxin concentration higher than 50 ug/l whereas resistant - tolerant cultivars tolerated concentrations up to 80 Ug/l.
   (iv) Evaluation of regenerated plants.
   All plants regenerated from the Callus were resistant to the pathogen in growth chamber.

**Work plan and time schedule (originally envisaged)**

Methodology

(a) Plantain collections will be concluded.

(b) Cytological and morphological characteristics of Nigerian plantains and bananas will be compared with those of other countries. Since many of the cultigens from these countries are in IITA collection in Onne, it will be possible to have fresh specimens in addition to descriptions in the literature. This work is already in progress.

(c) Cultures of Mycosphaerella spp. will be collected from several zones of the plantain growing areas of Nigeria and grown on different culture media such as (1) water agar, (2) Fries medium, (3) Soyabean broth and coconut milk (4) mycosel, and (5) mycophyl agar, in other to select which culture will give the greater yield for the different isolates.

(d) Using different plantain and banana cultigens serological studies and toxin characterization, the variability in the pathogen will be established.

(e) The potential of the use of the toxin in early screening and mass screening in cultures will be evaluated.

(f) Isolation and characterization of the toxin.
Chromatography: Descending paper chromatography will be carried out on oxalic acid Whatman No. 3mm chromatography paper suing (A) 1-propanol-ethyl acetate-water (B) ethyl acetate-acetic acid-formic acid-water (C) ethyl acetate-butanot-water as developing solvents (Kar et al. 1974).
Preparative and Quantitative Thin layer chromatography will be carried out using the method by Kar et al. 1974. Gas liquid partition chromatography of the trimethyl silycl derivative of the toxin will also be done.
The quantitative measurement of the toxin will be done by absorbance spectra method (Kar et al. 1975). The amount of toxin present in the filtrate will be expressed relative to a standard concentration curve prepared.

(g) The differential reactions of tissues to toxins of known concentrations by susceptible, tolerant, resistant, and immune cultigens will provide information on the utility of this technique on explants and hopefully on calluses.

(h) Cultures of Agrobacterium tumefaciens whose plasmids will be used as genetic carriers will be maintained in the laboratory.

(i) Basic study on callus production in plantain and bananas will be initiated.

(j) Plant transformation.
The bacterium, Agrobacterium tumefaciens will be used as a vector to transfer genetic material from resistant cultigens to the preferred clones. DNA extracted and purified will be mildly shared and treated with appropriate restriction enzymes. This will be allowed to anneal with similarly treated plasmids of A tumefaciens which will then be transferred back to the bacterium. The carrier bacterial will be allowed to multiply and used to infect the proliferating cultures resistant (transformed) plant cells will be selected using toxins. It is possible that a part of this aspect may be carried out in other laboratories more suited to genetic engineering. Such an arrangement has already been initiated.

## Work plan and time schedule

**(actual)**

**Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services training)**

1. Plant collection - Plant collection had already been carried out before this project started. It was funded by Federal Agricultural Coordinating Unit. This was from 1989 -1991. This was carried out by Fatunla and George Ude.
2. Cultures of the three species of leaf spots causing Sigatoka had already been established by 1990 -Blessed Okole.
3. The plan to use malate dehydrogenase, peroxidase, alcohol dehydrogenase, phosphoglucomutase, and triosephosphate isomerase. - by Akam.
4. Work plan on Tissue culture/screening for resistant tissues using toxin produced produced by the three mycosphaerella species. The protocol was jointly mapped out by Principal Investigator and Blessed Okole, a PH.D candidate. The workplan was taken to Germany for execution and by frequent interaction, the program was developed.
   (i) Pure cultures were developed using the ascospore discharge and spore pick technique (Natural, 1990; Foure 1990).
   (ii) Cultural and morphological characteristics of the three Mycosphaerella species. The growth media used were Potato dextroser Agar (PDA), Mycophil, V-8 juice, Malt extract, synthetic medium glucose, czapeks yeast, kind B, SNA, MID coconut milk. The colony colour, colony diameter, rate of colony growth pigment of colony, length of conidia, septation of conidia size and shape of spores.
   (iii) Toxin production and purification.
   Each of the three fungal species was grown in two litre Erlenmeyer flasks containing 800ml modified MID liquid medium. The medium was inoculated with two mycelial discs andshaken at 140 rpm for 28 - 30 days at 26°C with 12hrs of light in an incubator shaker. Equal volume of methanol was added to the fungal culture. The cultures were left for 24 hours at 4°C. The cultures were filtered through eight layers of cheese cloth and later through a 0.2 Um filter paper under suction. By concentration in a rotary evaporator at 38°C followed by exhaustive extraction with an equal volume of ethyl acetate/chloroform, the toxins were obtained.

5. Tissue culture propagation of Banana and Plantains
   Plant materials were from our collection at Ile-Ife and from the International Network for the Improvement of Bananas and Plantains (INIBAP) transit centre at Katholieke Universitat Leuven, Belgium.
   Shoot apices were prepared from the isolated corms of the cultigens after sterilizing in sodium hypochlorite (2.0%) for thirty minutes. The basal medium was Murashige and Skoog modified with basal salts and supplemented with sucrose Gelrite and a Vitamin mixture.
   Reducing agents (anti-oxidants) were used to prevent blackening of the tissues.
   After four weeks in the regeneration medium, rooted plantlets were transferred to the hardening medium which was composed of half-strength MS salts without growth regulators.
   Vigorous shoots 8 - 10 tall with roots were transferred to sterile soil for hardening.

6. Callus culture
   Callus cultures were produced from short tips approximately 5.0mm of each of the cultivars excised from the proliferating cultures and placed on modified MS medium and on Gamborg B-5 medium (1968). Auxins used included Dicamba (3,6 Dichloro-2-methoxybensoic acid), 2-4D and NAA. BAP and Zeatin cytokinin were also used.

7. Selection of Musa resistant cell lines.
   The partially purified (TLC) and HPLC toxins were diluted with molten (48°C) Gamborg B-5 medium containing Gelrite (1.5 g/L). The toxins were dilute ½, ¼, $^1/_8$, $^1/_{16}$ ($^1/_n$) with Gramborg B-5 medium containing Dicamba (30 Um)and BAP (20 UM).
   Cyclical selection (recurrent selection) was used by exposing call lines to increasing concentration levels of toxins, at each level surviving cells were allowed to recover - produce plantlets and from these plantlets callus was generated and then exposed to a higher level of toxin.

8. Use of Biochemical markers in identification of Plantain cultigens
   This aspect of the study is directed to indexing new lines from tissue cultures and to avoid unnecessary delay by waiting till morphological characters of the mature plant particularly the fruit and inflorescence.
   Leaf samples were obtained from the Teaching and Research farm, OAU, Ile-Ife, Nigeria. Crude protein and enzymes were extracted using appropriate extraction buffers, and homogenates centrifuged at 12,100 rpm at 4°C for 15 minutes. Polyacrylamide disc gel electrophoresis was used to determine the electrophoretic patterns in 12 plantain clones which were analysed in leaf protein, isozymes of peroxidese, tetrazolium oxidase and malate dehydrogenase.

9. Use of Morphological markers in identification of plantain cultigens.
   This aspect of the study is directed towards the assemblage of plant characters that could be useful in describing plantain cultigens in a gerplasm bank.
   (i) Stomatal studies in Banana and Plantain cultigens.
   Gum impressions were taken from the adaxial and abaxial leaf surfaces of seven cultigens. Three of these cultigens were plantain seedlings developed through meristem tissue culture while the remaining are mature cultigens. Photomicrograph of representative cultigens were also taken at X40 magnification. Samples were taken from the middle portion of the second leaf and the impressions were taken at the base middle and tip of each sample.
   (ii) Variation in Fruit types of plantain
   Data was collected on matured plantain bunches on the length of finger, diameter of finger, shape of fruit, total number of fingers per hand, hands per bunch, degree of curvature, number of fingers in the first basal arm and number of fingers on second basal arm.

## NETWORKING

(1) Federal Agricultural Coordinating Unit, Ibadan

(2) Blessed Okole (under Prof. F.a. Schluz
Technische University,
FG Phytomedizin, Lentzeallee
Berlin, Germany.

## PUBLICATIONS

(1) Akam C.A. (1993) Use of Biochemical Markers in identification of Plantain Cultigens - Unpublished M.Sc thesis

(2) Okole, B.N. (1993) Selection of Banana and Plantain (Musa spp) Tissues resistant to toxins produced by Mycosphaerella species using in vitro culture techniques. A report submitted to the Project Coordinator after two years study on the proposal in Germany.

(3) Nosiru, A.R. (1992) Stomatal Studies in Banana (Musa AAA group) and Plantains (Musa AB, AAB, ABB groups) Cultigens. B.Sc thesis

(4) Adedokun, A.A. (1994) Inflorescence as a Basis for Plantain Classification. B.Sc. thesis.

(5) Balogun, K.M. (1994) Variation in Fruit types of Plantain (Musa spp). B.Sc thesis.

(6) Afolabi, M.O. (1994) Inflorescence and fruit types of Bananas (Musa spp) in Nigeria. B.Sc thesis.

Part 4

## STATEMENT OF EXPENDITURES

| To be filled by ICGEB | | To be filled by the Affilliated Centre | |
|---|---|---|---|
| Budgets as per original proposal | | Summary of expenditures * | |
| 1) Capital Equipment | US$ _____ | 1) Capital Equipment | US$ _____ |
| 2) consumables | US$ _____ | 2) consumables | US$ _____ |
| 3) training | US$ _____ | 3) training | US$ _____ |
| 4) literature | US$ _____ | 4) literature | US$ _____ |
| 5) miscellaneous | US$ _____ | 5) miscellaneous | US$ _____ |
| **TOTAL GRANT** | **US$_____** | **TOTAL** | **US$_____** |

**Please itemize the following budget categories (if applicable)**

Capital equipment

| | |
|---|---|
| Deep Freezer (1) | 1750 |
| PC Computer (1) | 3000 |
| Repairs and spares | 2000 |
| Refrigerator (1) | 1500 |
| Dehumidifier (2) | 4000 |
| Cooled incubator (1) | 4000 |
| | 18250 |

Training (provide names, duration of training, host laborarory)

See attached Page 8

Literature

A lot of literature was photocopied and mailed to Nigeria from Germany. Similarly a lot of literature was obtained locally and are still being obtained.

* Please do not send invoices, receipts etc.; these should be kept by the Affilliated Centre for futere reference and sent to ICGEB upon request

Training (provide names, duration of training, host laboratory)

1. Blessed Okole - Department of Plant Science,
OAU, Ile-Ife of Nigeria
and
Technische University
FG Phytomedizin, Lentzeallee
Berlin, Germany

Worked on Tissue culture and Breeding for resistance - for three years (Doctoral Student) Duration of Training 2 years

2. George Ude - Department of Plant Science
OAU, Ile-Ife, Nigeria
and
IITA, Ibadan

Worked on Plant Collection and variation in morphological traits Duration of Training - for 2 years (Doctoral student)

3. Cletus Akam - Department of Plant Science
OAU, Ile-Ife, Nigeria

Obtained his masters in Plant Science working fully on this project - 2 years

4. 1. NDSIRU, A, 2. Afolabi, M.O. 3. Balogun K.M. 4. Adedokun, A.A
Department of Plant Science
OAU, Ile-Ife