FINAL REPORT

ON

Genotyping (Manitoba/CIAT)
Centre File: 3-P-83-1031-01/2

THE UNIVERSITY OF MANITOBA

FOOD SCIENCE DEPARTMENT

Winnipeg, Manitoba
Canada R3T 2N2

(204) 474-9621 (General Office)
(204) 474-9601 (Head)
(204) 983-5785 (Telefax)

January 11, 1988

Dr. H. Zandstra
Director, AFNS Division
International Development Research Centre
P.O. Box 8500
Ottawa, Ontario
K1G 3H9

Dear Sir:

Re: **Genotyping (Manitoba/CIAT)**
Centre File: 3-P-83-1031-01/02

I have the honour to submit herewith the Final Report on the subject project.

The financial support of IDRC and the interest and cooperation of all concerned at IDRC and CIAT is gratefully acknowledged. On behalf of Drs. Laing and Roca and myself, I would like to extend special tribute to Dr. Hussain and Mr. Ramirez for their hard work and perseverance which contributed immensely to the success of the project. Finally, my sincere thanks to Mrs. Heather Delorme for her patient stenographic services.

Yours sincerely,

W. Bushuk

W. Bushuk
NSERC Research Professor

WB/hd

cc. M. Loevinsohn
G. Hawtin
N. Mateo
S. Dillabough
J.L. Nickel
D.R. Laing
W.M. Roca
H. Ramirez
A. Hussain
Office of Res. Admin.
R.C. McGinnis
E.D. Murray

FINAL REPORT

TO: Agriculture, Food and Nutrition Sciences Division,
International Development Research Centre

PROJECT TITLE:

Electrophoretic characterization of genotypes in CIAT collections of cassava (Manihot esculenta Crantz), field bean (Phaseolus vulgaris L.), and forage legumes (Centrosema, Desmodium and Stylosanthes)
Centre File 3-P-83-1031-01/02

PROJECT LEADERS:

Dr. W. Bushuk
Food Science Department
University of Manitoba
Winnipeg, MB, Canada R3T 2N2

Dr. D.R. Laing
Deputy Director
Centro Internacional
de Agricultura Tropical
Cali, Colombia

COORDINATOR (CIAT)

Dr. W.M. Roca
Head, Biotechnology Research Unit
Centro Internacional de Agricultura Tropical
Apartado Aereo 6713
Cali, Colombia

INVESTIGATORS

Dr. Akhtar Hussain
Research Associate
Food Science Department
University of Manitoba
Winnipeg, MB, Canada R3T 2N2

Mr. H. Ramirez
Research Assistant
Centro Internacional
de Agricultura Tropical
Apartado Aereo 6713
Cali, Colombia

WINNIPEG, CANADA

December 1, 1987

CONTENTS

I. OBJECTIVES

Continuous addition of new varieties, limited space for germplasm conservation, maintenance costs, insufficient or missing data are the elements that demanded an accurate and precise germplasm identification system. Accordingly the objectives of this project were set primarily to develop objective techniques which could simplify the complex nature of morphological descriptors. It was anticipated that with today's powerful methods of analysis, protein characters can be useful to plant breeders and taxonomists in solving difficult problems for which morphological comparison have intrinsic limitations.

The overall project approach was aimed at:

1. Developing methodologies for routine characterization of genotypes in CIAT commodity crops (viz. cassava, field beans and the pasture legumes).

2. Improving and updating existing data on genotypes held in the world collections at CIAT.

3. Screening and verifying genotypes of parental and filial populations.

4. Fingerprinting agronomically important traits with a view to upgrade seed quality.

5. Computerizing the electrophoretic data for easy access, comparison and comments.

To achieve the aforementioned goals, the following variables were recognized as worth considering:

1. Selection of appropriate tissue sample for particular protein fraction.

2. Selection of extraction media and electrophoretic procedures best suited to obtain maximum intercultivar discrimination.

3. Optimization of selected procedures to maintain high standards of electrophoregrams.

4. Computerization of electrophoresis information to facilitate cataloging and verification of new genotypes.

5. Publication of results in scientific journals to make the information available to all interested parties.

II. EXECUTIVE SUMMARY

The major thrust of this project has been the development of methodologies. Details of these methodologies are dealt with individually in appropriate sections. Procedures have been developed which can discriminate cultivars of cassava (Manihot esculenta), field bean (Phaseolus vulgaris) and the pasture legumes (Desmodium ovalifolium, Centrosema macrocarpum, C. pubescens, Stylosanthes capitata and Pueraria phaseoloides). Acidic PAGE (polyacrylamide gel electrophoresis) patterns can also distinguish seeds of cow pea (Vigna angiculata) and field bean (P. vulgaris) which sometimes are difficult to differentiate by seed morphology.

The techniques that have been developed can be used for other purposes, i) in biochemical genetics e.g. in locating the real parentage of an hybrid, and ii) in planning of some crosses for plant breeding. Two individuals with entirely different electrophoregram types can be crossed to determine the inheritance of certain protein characters. The purity of a particular collection can be readily checked by using the same electrophoretic procedure for randomly selected seeds within one collection. Any heterogenity in the banding pattern would indicate an admixture. Based on analyses of limited number of accessions, a high degree of chemical variation relative to the visual or structural variations, was observed. In some crops protein bands can be directly related to agronomic end-use quality characteristics e.g. phaseolins, arcelins, luster of seed coat, cooking quality, etc. Techniques developed for the pasture legumes helped in verification of some of the taxonomic problems encountered by breeders in these crops. The germplasm Unit of Tropical Pasture Program will further evaluate their collections and will use the data in planning breeding strategies.

In addition, isoenzyme electrophoresis has proved useful for early selection of somaclones regenerated from Stylosanthes and cassava tissue cultures. The technique can be used to cultures obtained by protoplast fusion of Stylosanthes species. As well, isozymes are useful markers for somatic embryogenesis in cassava.

Research collaboration has benefited both parties, the University of Manitoba in terms of publications and in establishing further cooperation between the two, and Centro Internacional de Agricultura Tropical (CIAT) in transfer of technology, training and on site establishment of electrophoresis facility. CIAT, among the international agricultural research centres, now leads in the area of electrophoresis.

c. **MISS LUZ MARINA TELLO,** Universidad del Valle. To work on "Characterizacion de embriogenesis somatica mediante electroforesis de isoenzimas en cassava" under the direction of Drs. L. Szabados and W. M. Roca.

2. **Trainees**

MS. ALFORA ESTELLA GONZALEZ	**(COLOMBIA)**
MS. MARIA DEL CARMEN MENENDEZ	**(ARGENTINA)**
MR. RAYMUNDO CRUZ	**(CUBA)**
MR. GILLERMO DELGADO	**(PERU)**
MS. ROSALIA VELASOVEZ	**(VENZUELA)**
MS. MARIBEL TREJO	**(VENZUELA)**
MS. LIGIA CARMENZA MUNOZ	**(COLOMBIA)**
MS. DIANA ISABEL ARIAS	**(COLOMBIA)**
MR. ROBERTO SIMMONOS	**(COLOMBIA)**
MS. MARTHA CATANO	**(COLOMBIA)**
MS. MARIA ESPERANZA SOTO	**(COLOMBIA)**

3. **Programs using the research facility at BRU**

a.	Genetic Resources Unit	(Conservation & classification)
b.	Bean Program	(Breeding & pathology)
c.	Cassava Program	(Breeding & physiology)
d.	Tropical Pasture Program	(Breeding & Taxonomy)
e.	Rice Program	(Pathology & Breeding)
f.	Special project; IBPGR-CIAT at BRU	(Characterization and monitoring stability of in vitro collection of cassava)

IV. TECHNICAL PROGRESS

A. INTRODUCTION

CIAT has a global mandate for the conservation, evaluation, exchange and cataloging of the world collection of cassava, field bean and tropical pastures. The germplasm of these crops was collected throughout their main centres of diversity by cooperation of various national and international agencies. They came from areas with distinct agroclimatic conditions and from remote pockets where loss of genetic material was threatened. These germplasm collections are clustered using traditional taxonomic techniques where morphology has been the major source of information. Today we have learnt that morphological features may vary depending on the biotic and abiotic stresses. It is very likely that this modified morphotype may not be in line with the expected descriptors. Moreover the boundaries between varieties within one species are so ambiguous that clear cut demarcation is hard to establish. Since morphological characters have a chemical foundation, it was therefore considered proper to obtain chemical data. Chemical data on the average would be relatively closer to gene(s) which are least affected by the environmental stresses. To acquire such data especially pertaining to crops of tropical origins, this project was initiated with the financial support from IDRC.

B. CASSAVA (Manihot esculenta Crantz)

With the expanding world collection of cassava it was becoming unmanageable to identify and catalogue varieties using traditional ways of classification. It was therefore felt necessary to devise methodology which could routinely be applied to accurately evaluate the clones of cassava.

1. Development of methodology

As the protein contents of this particular crop were extremely low, only few experiments were carried out using total proteins and they did not yield encouraging results. Naturally the attention was diverted towards the study of isoenzymes. After extensive research exploring 16 enzymes in five different tissues using five different schemes we were able to conclude the following:

a. Loci showing polymorphism were noticed in the enzymes esterase, diaphorase, acid phosphatase, peroxidase and glutamate oxaloacetate transaminase.

b. Enzyme esterase out of the above group presented the most intercultivar discrimination.

c. Root tissue of 3-4 wks old cassava plants was found to be the best source tissue of the enzymes which provided most discrimination.

d. The running buffer Tris-borate (0.05M) pH 9.0 and 12% acrylamide gel provided the optimal resolution of enzymes.

2. Application of developed methodology

This methodology was initially tested with cassava clones from different climatic regions and later tested with clones from the same agroclimatic region. In both cases results showed 100% interclone differences.

This methodology was also used to help germplasm management, to characterize selected clones and to monitor genetic stability of in vivo and in vitro material.

About 219 clones classified into 78 morphotypes were investigated to check duplication if any. About 40% of clones turned out to be duplicates. This exercise certainly helped in management of the germplasm.

Clones of MCol 1505 propagated through meristem-tip micropropagation, in vitro conservation, somatic embryogenesis, stake propagation and bud propagation were evaluated using esterase patterns. Results demonstrated the stability of the genotypes under these treatments.

Effect of in vitro storage was evaluated by analyzing the material raised from shoot tips previously stored in liquid nitrogen and the fresh material obtained through propagation of fresh stakes. The patterns obtained for the two treatments did not show differences indicating persistence of the patterns.

This procedure has also been adapted by a collaborative project between CIAT and IBPGR for the characterization of various morphotypes (100 clones). The objective is to establish a pilot in vitro active gene bank (IVAG) in CIAT.

To remove roots from the in vitro culture collection means destruction of cultures. Sometimes it is difficult to sacrifice plantlets. Thus 3/4 of shoot portion was used as the source tissue instead of roots and two enzyme system viz. diaphorase and esterase was used to evaluate in vitro germplasm collection. Rest of the plantlet can resume its growth without affecting the totality of collection.

This technique is currently in use to study the stability of enzyme characters under different conditions such as prolonged culturing, virus elimination, growing media, heat treatment, and frequent subculturing.

Esterase patterns of the roots from stakes and roots from the in vitro plants of the same cultivar were found to be different. It will be useful to associate this change of patterns.

C. FIELD BEAN (Phaseolus vulgaris L.)

1. Development of methodology

Following the success with cassava, preliminary research with beans was initiated with the investigation of various enzymes. Twelve enzymes were studied in different tissues, using various extraction schemes and buffers. After about 200 observations we learnt that bulk of the enzymes were localized in the growing regions of 3 week old seedlings. To achieve 100% discrimination based on isozymes, even in genetically diverse samples was not possible. Enzymes esterase, peroxidase, alcohol dehydrogenase, diaphorase, acid phosphatase and shikimate dehydrogenase were observed to contain polymorphic loci. Diaphorase was best extracted from root tips of 2-3 wk seedlings and dry mature seed was a good source of alcohol dehydrogenase. Combination of these enzymes could cluster the samples into groups but could not discriminate individual genotypes. The whole procedure is laborious, time consuming and uses expensive substrates. Moreover it was difficult to obtain plants of exact physiological age as some varieties germinated earlier or grew faster than others. Also enzyme extracts seem to have a very short shelf life as compared to protein extracts. For these reasons the work involving isozymes was discontinued and attention was focussed on proteins which are independent of development stages, more stable in extracted form and represent a relatively broader genetic base. Moreover this research was aimed at to develop techniques which are relatively less complex and rapid but with most discriminative power.

Investigations began with extracts of seed protein in various solvents. In the successful procedure residue obtained after sequential washing (salt, alcohol, acetic acid) was extracted in Tris-HCl pH 8.3 buffer containing PVP-40, Triton X100, SDS, 2-mercaptoethanol and glycerol. SDS PAGE of this residual protein fraction showed intercultivar differences. This separation was initially carried out in 10% SDS gels but bands useful in discrimination were diffuse and shadowy. Increase in acrylamide concentration from 10% to 15% ractified these problems. This procedure however required 10 hours of electrophoresis in addition to sample preparation. To fit this time scale in the daily schedule was not easy and the need for a relatively rapid procedure was iminent. IEF and acid PAGE systems were known to be quite fast in separating plant proteins. IEF would use expensive ampholines, special chamber and expensive power source. Thus it was decided to try acid PAGE system prior to IEF. Out of the eight different types of extraction solutions and buffers

employed to extract proteins for acid PAGE, 0.1M acetic acid containing 1% 2-mercaptoethanol produced encouraging results. Having selected the extraction solution, electrophoretic conditions were modified to obtain sharpness in protein characters. The two procedures using SDS PAGE and acid PAGE have been published in Euphytica.

Methodology of acid PAGE was further modified by introducing 5% sucrose in the gel and reducing current from 40mA to 30mA. One hour incubation at 40°C of seed meal in the extraction solution facilitated protein extraction. Removal of seed coat prior to preparation of seed meal reduced the amount of tannins which otherwise would interfere with resolution of protein.

2. Application of methodology

Initially this technique (acid PAGE) was tested on accessions with similar seed and plant morphology. These morphologically similar accessions showed more electrophoretic variation than similarities. Source of these heterogeneities among the electrophoregrams of morphologically similar seeds is unknown but these variations could have been incorporated as a result of outcrossing and/or residual heterozygosity. Genetic Resources Unit of CIAT then supplied accessions originating from various parts of the world identified as one variety. Protein analyses of such samples showed interaccession variation within a single cultivar. Different types of electrophoretic patterns observed within one variety are listed below:

Number of accessions analysed	Cultivar	Number of banding patterns
24	Flor de Mayo	9
9	Cargamento	5
2	Rojo-70	2
3	Cornell	2
3	Compuesto	3
5	Aurora	5
2	Venzuela	2
2	Kaboon	1
2	Vermelho	2
2	Constanza I	2
2	Pompadour	2
2	Canario Divex	1
2	10233-3M-11	1
2	Great northern	1

This led to a possibility that the particular accessions may not be chemically pure as the seed meal was prepared using bulk sample of 15 seeds. Further study was thus directed to investigate the uniformity of the seeds within collections. Interseed variations were observed in about 25% of the accessions,

in contrast to the 4% visible variation. In most accessions variation in protein character was restricted to one out of ten seeds. Also this variation was limited to one or two bands difference in most cases. This variation observed in the electrophoregrams may not be related to agronomic characteristics but the differences definitely indicate that the accession is impure. Some accessions among the tested, showed higher degree of variability. The seeds of these accessions were reexamined for visual uniformity. Accessions G04354 and G05772 which showed 4 and 6 types of electrophoretic patterns, respectively, also showed great diversity in seed morphology. Accession G04354 was separated into the following seed morphotypes:

i. large elipsoidal brilliant
ii. medium rounded brilliant
iii. small rounded dull and
iv. small rounded brilliant

Two seeds from each types were analyzed separately using acid PAGE. Each morphotype had a unique pattern. Looking at the visible variables in the seed types it was possible to link a particular protein band to brilliance of seed coat. This band was absent in seeds with dull seed coat. Luster of the seed coat is very important for evaluation of cooking quality. Seed with brilliant seed coat usually is less permeable as compared to the seed with dull luster (presumably these contain less wax). More research is needed to link this trait to a particular protein.

To estimate heterozygosity, seeds from different plants of the same variety, different pods of the same plant and seeds from the same pod were analyzed using acid PAGE procedure. Cultivars ABA2 and AB57 showed no differences between the seed sampled. These varieties had been selfed for 15 years. Thus varieties selfed for longer periods may attain homozygosity.

A totally different protein pattern appearing among the electrophoregrams of similar seeds would mean physical contamination rather than outcrossing. These results therefore imply that interseed variation analyses may be necessary where purity of genotype is crucial.

Acid PAGE methodology was also applied in biochemical cytogenetics of beans. In some instances the paternal or maternal parentage is not known. To determine the potential male parent in a cross, seeds from hybrids, mother and from possible fathers were analyzed in acid gel. Protein characters present in the hybrid electrophoregrams can easily decide who the possible parents could be. Using this procedure the actual male and female parents were identified.

3. Development of acid PAGE procedure for phaseolin characters

Phaseolins (globulins) have gained importance over the past few years as to determine geographic origin and nutritional quality of field beans. Extensive research is being carried out using procedures which are lengthy and do not provide good resolution. An alternative procedure based on acid PAGE was developed. This system extracts phaseolins in 0.4M NaCl containing 33% mercaptoethanol. It should however be pointed out that quantities of NaCl and ME are very crucial for extraction of phaseolin types. A slight increase in NaCl content extracts more protein whereas a slight increase in mercaptoethanol give extracts that produce a smaller number of bands on electrophoresis.

4. Confirmation of seed types

Sometimes it is difficult to distinguish between the seeds of cow pea (Vigna angiculata) and field bean (Phaseolus vulgaris) by seed morphology. Seeds of these species have to be grown for at least ten days to discriminate the species. Using the developed electrophoretic procedure, it is easy to determine whether the seed belongs to Phaseolus or Vigna. Presence of two strong globulin bands would place the seed into Phaseolus and their absence would eliminate from this genus.

5. Recognition of reference band

To normalize data from several gels and to determine relative mobilities it is necessary to have some sort of reference system. For high precision, it is necessary to run reference extracts on both sides of the gel. In field beans, we have identified one species-specific band which has appeared in all accessions examined so far. An arbitrary mobility number of 80 has been given to this band. Other bands can be easily identified by mobility number relative to band 80.

In conclusion, much of the information on electrophoretic properties of field bean proteins reported herein is potentially applicable to research on bean improvement in addition to variety discrimination and identification.

D. **PASTURE LEGUMES**

1. Desmodium ovalifolium

The work with pasture legumes initially included four genera viz. Desmodium, Centrosema, Stylosanthes and Zornia. During on site review, Zornia was dropped from the list and replaced with Pueraria. Research in this area began with development

of a procedure for cultivar identification of Desmodium ovalifolium. Cultivars of Desmodium were selected based on diverse plant morphology; genetic constitution of the selected accessions was unknown. From our experience with other legumes we felt unnecessary to investigate isozymes as potential markers for cultivar identification. Nevertheless some enzymes such as esterase, peroxidase, glutamate oxalo acetate transaminase and acid phosphatase were checked in seed tissue of Desmodium cultivars. These enzymes failed to provide complete discrimination of tested samples. Analyses of different tissues revealed that root tissue of D. ovalifolium is a good source of enzymes but even root extracts did not yield satisfactory discrimination between cultivars.

The most promising results were obtained using seed inactive proteins as genotypes markers. Tissue of mature dry seed was preferred as the source of protein because it represents the final stage of plant development. Several extraction procedures were investigated. The 1% SDS extract proved useful in cultivar identification but it contained substantial amounts of phenolics and/or of high molecular weight substances. These contaminants remained in the slot and prevented other proteins from entering the gel. Certain amount of precipitation in the gel was also observed. As a result the electrophoregram contained distorted bands. We tried to resolve this problem by precipitating out some of the large molecular weight protein by the addition of ammonium sulfate. We realized that by using salt precipitation we added more problems. Removal of the ammonium salt which interfered with electrophoresis was required. Several days of dialysis was necessary to get rid of the sulfate and the final extract was too dilute to give a satisfactory pattern. To achieve exact concentration of ammonium sulfate in the crude protein extract was very difficult. Thus we resolved this problem by another technique. Some of the interfering proteins were removed by immersing the crude extract in boiling water for not more than 2 min. Heat unstable substances which precipitated were removed by centrifugation. Electrophoresis of the supernatant provided satisfactory intercultivar discrimination but the tailing effect was still present. The extract was therefore acidified with aluminum lactate buffer pH 3.1 (1:1 v/v) from pH 8 to pH 6. This step precipitated some proteins. The precipitated proteins were removed by centrifugation. The residue washed twice with distilled water was dissolved in the minimal volume of the original extracting solution. This solution when resolved in a basic gel (without SDS) gave patterns that showed sufficient intercultivar variation necessary for discrimination and identification. The procedure was published in Canadian Journal of Plant Science and is being used for further evaluation of CIAT collection.

2. Centrosema

Various systems and a variety of extraction buffers were tested in developing the genotype identification procedure for Centrosema cultivars. The basic system in which the seed proteins were extracted in Tris-HCl pH 8.3 provided sufficient interspecies discrimination. After testing the entire range of pH of this buffer, it was concluded that Tris-HCl pH 7.5 was the best for our purpose. This procedure however failed to show differences when applied to various cultivars within a single species of Centrosema. Accordingly further work was done using an acid PAGE procedure. After preliminary screening of the extraction buffers, sodium acetate solution, pH 5.8 gave satisfactory extracts. Two hours of incubation at 40°C further facilitated the extraction of proteins. Other conditions of electrophoresis, viz. acrylamide conceneration and current settings, were modified to obtain improved pattern quality and band resolution. This procedure (extraction of proteins with sodium acetate buffer pH 5.8, and separation in 10% acid gel at temperature of 20°C and a current of 15 mA) clearly discriminated between species as well as between cultivars within species of Centrosema.

Electrophoretic analysis of various species of Centrosema showed that the two accessions of C. acutifolium did not exhibit normal interspecies homology in band patterns. This suggest that the two cultivars may be of different species or subspecies of acutifolium as proposed by some of the taxonomists. Within the species C. pubescens and C. macrocarpum some accessions gave electrophoregrams that are quite different from the common banding patterns. Later it was observed that accessions with drastically different patterns had diverse plant morphology. Thus both morphological and protein characters can help to revise the classification of doubtful accessions.

The procedure developed by this research has been applied to resolve several other problems in the taxonomy of Centrosema (see publication No. 5).

3. Stylosanthes capitata

Research on Stylosanthes capitata followed the pattern established for other crops investigated in this project. Again, isozyme patterns failed to give satisfactory intercultivar discrimination. Inactive proteins were therefore used as genotypic markers.

After extensive research, a technique was developed which gave acceptable discrimination of a small number of selected cultivars. The procedure used Tris-HCl (pH 8.3) extracts and 12% acrylamide gels at pH 8.8 (see publication No. 6). The procedure is being further evaluated (beyond the funded period of the project) by analyzing additional accessions available at CIAT.

Furthermore, it is being used to resolve problems relating to accession identity and seed purity encountered by CIAT plant breeders.

4. Pueraria phaseoloides

A new forage legume Pueraria phaseoloides which replaced Zornia in the project was the next species to be investigated. Attention was focussed directly on analysis of inactive proteins by acid PAGE. Several different extracting solutions were tested. In the successful procedure, seed proteins are extracted in 5M acetic acid and separated in 8% gel at pH 3.1 with aluminum lactate as the running buffer. Ten cultivars selected on the basis of diverse plant morphology showed unique protein patterns. Protein bands in a region closer to origin (perhaps with high molecular weight and more +ive net charge) could characterize intercultivar variations. Bands in other regions of the gel may be useful for determing the degree of pattern homology.

5. Brachiaria

Brachiaria, the most important pasture grass in the tropics, was left out in the original list. With the co-operation of the CIAT Tropical Pasture Program, work has begun on the investigation of this particular genera with a view to characterize both interspecific and intervarietal differences. Esterase isozymes have been found useful in obtaining sufficient discrimination. The procedure is currently being used to ascertain the presence of electrophoretic patterns which indicates a combination of parental bands in selections from specific crosses. Since Brachiaria is characterized by the occurrence of apomixis, particular patterns would indicate the occurrence of sexual recombination. This would allow the breeders to tap the diversity present in apomictic materials. This research is continuing at CIAT beyond the funded point of the project.

E. ADDITIONAL RESEARCH

1. Rice

To meet the growing demand of the Rice Program some preliminary work was done on the development of an electrophoretic procedure for identification of rice cultivars. Of the enzymes present in 3-4 week seedlings, esterase provided greatest discrimination. The heterogenity however was restricted to the minor bands. Resolution of these bands in 10% polyacrylamide gel was far superior than in 11% starch.

Several extracts of inactive seed proteins were examined as potential genotypic markers. Sodium chloride extracts did not discriminate the 10 cultivars that were analyzed. Aluminum lactate (pH 3.1) extracts showed sufficient discrimination. Densitometer recordings of the electrophoregrams showed differences between cultivars which otherwise are difficult to see. Extracts in 5M acetic acid were also useful in cultivar identification. The methodology based on this extract is currently being used in attempt to identify a protein character responsible for (or linked to) resistance to "Hoja Blanca" disease. This work is in its early stages; results are very encouraging and the work is continuing.

2. Cowpea

Some preliminary work was carried out to identify cultivars of Vigna angiculata. Using the acid PAGE procedure outlined for Phaseolus vulgaris, cultivars of cowpea gave unique patterns. Further work on this crop will be carried out depending on potential interest from other IARCs.

3. Biochemical genetics

Some progress has been made on the use of electrophoresis to study the inheritance of certain protein and isozymes characters.

In beans, most variations lie in the central part of gel which usually contain bands of arcelins and not phaseolin. Based on arcelin homology cultiars of beans will be grouped for use in cross breeding. In case of cassava the methodology described earlier is currently in use in an IBGR collaborative project on the maintenance of genetic stability of in vitro varieties. In this context appropriate crosses will be made to study the inheritance of esterase isozymes.

F. DATA PROCESSING AND STORAGE

Before taking photographs of the electrophoretic patterns, the stained gel was washed with soapy water and rinsed twice with distilled water. The stain particles adhered to the gel were removed from both surfaces by cotton swabs (cotton buds). The gel was chilled (-10°C) at least for 10 min in order to enhance sharpness of the bands. The cool gel was placed on a clean dry glass plate on top of a light source for photographs using an SLR camera (Pentax). Use of a yellow filter improved the quality of the picture. Kodak technical pan X film produced negatives of good contrast. High contrast Kodak paper was used to make prints for filing and publication.

Photographs were used to compare and analyze a small number of electrophoregrams. In the work on beans band 80 which was present in all the accessions that were examined, was used as the reference for normalizing small variations between gels. For cassava, it was necessary to run two references on both sides of the gel and calibration of mobilities was difficult. To overcome this problem an LKB Ultra Scan XL Laser Densitometer with IBM compatible software, recently acquired by CIAT, was used to file profiles graphics, number of peaks, peak position, peak height, peak area and relative peak area.

Currently, CIAT scientists are in the process adapting the LKB instrument for storage and analysis (vis a vis variety identification) of electrophoretic patterns. Computer software for various applications has been ordered. This part of the project will continue beyond the funded period of the project.

V. PUBLICATIONS

1. **Hussain, A., W. Bushuk, H. Ramirez and W. Roca,** 1986. Field bean (Phaseolus vulgaris L.) cultivar identification by electrophoregrams of cotyledon storage proteins. Euphytica 35:729-732.

2. **Hussain, A., W. Bushuk, H. Ramirez and W. Roca,** 1987. Identifcation of cassava (Manihot esculenta Crantz) cultivars by electrophoretic patterns of esterase isozymes. Seed Sci. Technol. 15:19-22.

3. **Ramirez, H., A. Hussain, W. Roca and W. Bushuk,** 1987. Isozyme electrophoregrams of sixteen enzymes in five tissues of cassava (Manihot esculenta Crantz) varieties. Euphytica 36:39-48.

4. **Hussain, A., H. Ramirez, W. Bushuk and W. Roca,** 1987. Identification of cultivars of forage legume (Desmodium ovalifolium Guill et Perrr.) by their electrophoretic patterns. Can. J. Plant Sci. 67:713-717.

5. **Hussain, A., H. Ramirez, W. Bushuk and W.M. Roca,** 1987. Identification of cultivars of the pasture legumes (Centrosema macrocarpum, C. pubescens and C. sp.n.) by acid polyacrylamide gel electrophoresis of cotyledon storage proteins. Euphytica (in press).

6. **Hussain, A., H. Ramirez, W. Bushuk and W.M. Roca,** 1987. Identification of Stylosanthes capitata by polyacrylamide gel electrophoresis of seed proteins. Euphytica (in press).

7. **Hussain, A., H. Ramirez, W. Bushuk and W.M. Roca,** 1987. Interaccession variability of electrophoregrams of cotyledon protein of field bean (Phaseolus vulgaris L.) Euphytica (submitted).

8. **Hussain, A., M.T. Espinel, W.M. Roca and W. Bushuk,** 1987. Acid PAGE procedure for evaluation of phaseolin types in Phaseolus vulgaris L. Euphytica (in press).

9. **Hussain, A., W. Bushuk and W.M. Roca,** 1987. Identification of cultivars of the forage legume Pueraria phaseoloides by electrophoretic patterns of storage proteins. Euphytica (submitted).

10. **Hussain, A., H. Ramirez and W.M. Roca,** 1986. Manual Practico para la deteccion electroforetica de isoenzimas y otras proteinas.

    Documento de Trabajo No. 19,
    publ. Centro Internacional de Agricultura Tropical,
    Cali, Colombia.

11. **Hussain, A., W. Bushuk, H. Ramirez and W.M. Roca,** 1987. Polyacrylamide gel electrophoresis procedures for cultivar identification of field bean, cassava and the pasture legumes.

Working Document No. 22,/Univ. Man. Food Sci. Publ. 115. publ. Centro Internacional de Agricultura Tropical, Cali, Colombia.

12. **Hussain, A., W. Bushuk, H. Ramirez and W.M. Roca,** 1987. A Practical Guide for electrophoretic analysis of isoenzymes and proteins in cassava, field bean and forage legumes. (in press).

VI. SUMMARY OF ACHIEVEMENTS

1. Essentially all of the objectives of this project as stated in the original proposal have been achieved. The minor exceptions are: i) The forage legume **Zornia** was not investigated and the forage legume **Pueraria** was substituted because of its current importance. ii) The use of the computer for storage and analysis of electrophoretic data has not been developed to the stage where it can be applied routinely; this part of the project will continue beyond the period of funding.

2. Genotypes of **cassava (Manihot esculenta Crantz)** can be discriminated on the basis of their unique (genotypic) isozyme patterns. Methods based on polyacrylamide and starch gels have been developed. Of the sixteen enzymes investigated, esterase gave the best discrimination. Results of this part of the project has been published in two scientific papers (No. 2 and 3).

3. Cultivars of **field beans (Phaseolus vulgaris)** can be discriminated and identified on the basis of the electrophoregrams obtained by acidic PAGE (polyacrylamide gel electrophoresis under acidic pH conditions) of an acetic acid extract of cotyledon proteins. In addition to cultivar discrimination, the method was used in the following studies: i) Determination of the purity of the seed in a single accession. ii) Relationship between seed morphology and electrophoretic patterns. iii) Intercultivar variability of phaseolins. iv) Discrimination between field bean (**Phaseolus vulgaris**) and cow pea (**Vigna**) This part of the project produced three scientific papers (No. 1, 7 and 8).

4. Cultivars of the forage legume **Desmodium ovalifolium** Guill et Puerr., were discriminated by basic PAGE of a characteristic protein fraction prepared from total seed protein (publication No. 4).

5. Cultivars of the **Centrosema** species were discriminated on the basis of the electrophoregrams of sodium acetate solution extracts of seed proteins obtained by acidic PAGE (publication No. 5).

6. Genotypes of **Stylosanthes capitata** were identified by the electrophoregrams of seed protein extracts in Tris-Hcl buffer (pH 8.3) obtained by basic PAGE (publication No. 6).

7. Genotypes of **Pueraria phaseoloides** were discriminated on the basis of electrophoregrams of acetic acid extracts of total seed protein obtained by acidic PAGE (publication No. 9).

8. The equipment and methodologies developed in this project were used in preliminary experiments on the discrimination of

genotypes of **Brachiaria** (a tropical pasture grass), cowpea, and rice. These crops are of interest to CIAT but were not part of the project.

9. A large number of students and technicians were trained in the use of electrophoresis for crop research.

10. In addition to the scientific publications the research produced three methods manuals written in Spanish and English (publications No. 10, 11 and 12).

11. A viable research and service laboratory based on various types of electrophoresis has been established at CIAT. It is anticipated that this laboratory will make a substantive contribution to the programs of CIAT and those of other national and international institutions.

VII. CONFERENCES/REVIEWS

May 5-9, 1985 — International Symposium on Biochemical Approaches to identification of cultivars and evaluation of their properties. Braunschweig, West Germany.

Part of cassava work presented.

Internal Reviews:

November 1985: W.M. Roca, W. Bushuk, A. Hussain and H. Ramirez: Discussion and further planning. University of Manitoba.

March 1987: W. Bushuk, W.M. Roca, A. Hussain and H. Ramirez presented an overview of the project: Two days seminar was attended by Deputy Director, CIAT, various heads of units/programs and IBPGR representatives.

VIII. SUGGESTED FURTHER RESEARCH

1. Continuation of electrophoretic analysis of CIAT germplasm collections with a view to cluster accessions on the basis of both morphological and chemical characters.

2. Studies of the inheritance of protein characters and their linkage with traits of agronomic importance.

3. Study of the effect agroclimatic conditions on electrophoretic patterns.

4. Investigation of marker protein(s) in tropical pastures.

5. Research on protein(s) responsible for viability or deterioration of bean seeds. Beans with lighter seed coat seem to deteriorate faster than those with darker seed coat.

6. Research on marker protein(s) which can determine the geographical location of centres of origin of field bean.

IX. FINANCIAL STATEMENTS

Details on the use of the University of Manitoba portion of the funds provided for the project by IDRC will be submitted by the Office of Budgets and Grants of the University of Manitoba in accordance with standard reporting procedures. Presumably the same will be done by appropriate officials of CIAT for the portion of the budget administered by that institution.