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PROJECTS SUPPORTED BY COLCIENCIAS

1991 - 1997

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PREFACE

Biotechnology, along with electronics, telecommunications, information sciences and new materials is one of the high technologies that appears as an option for solving many of the basic problems of human health, agriculture, cattle raising, food and environment in the next century. Its potential for the development of new products and services encompasses various fields such as: disease-free plant production, genetic transformation of animal and plant species to produce disease-, pest- or environment-resistant varieties, identification techniques for human, animal or plant pathogens, alternatives for food and medicine production, and improvements in waste disposal, among other possibilities.

Biotechnology is an activity that requires deep and extensive knowledge, and interdisciplinary work, in order to lead to a better quality of life.

Colombia has not stayed outside from biotechnology developments and the investments made during the 80s and 90s are starting to yield results, particularly in the agricultural and environmental fields.

This book reflects the state of the art in biotechnology research developed with the sponsorship of Colciencias, in different fields, but especially in the agricultural one. It is a response to the scientific community and the research and academic development groups, which work in that area.

Results have been achieved in the following areas: a.Biopesticides based on *Bacillus thuringiensis* and *Beauveria bassiana*. These results promoted the formation of a Colombian research network in this field, an the commercial production of the biopesticides. b.Programs aiming to the obtention of plantain and banana plants resistant to yellow and black sigatoka. c.Virus identification and obtention of passion fruit and potato virus free plants. d.Detection of several CTV (Citrus tristeza closterovirus) isolations, which is one of the limitant factors for the production of citrus fruits. e.Use of molecular biology techniques for plant virus and fungi diagnose, such as *Fusarium oxysporum* f. sp, *dianthi*, as one of the main problems in carnation plantation. f.Development of systems for producing transgenic coffee plants. In human health, there have been results as: a.Commercial production of diagnose kits based on monoclonal antibodies. b.Development of commercial products for antigen HLA-DR typification, replacing the traditional serologic methods. c.Use of hybridation in situ for detection of genetic annomalies in afected and asymptomatic individuals, as a step for disease prevention and diagnose. d.Production of the main alergen from *Blomia acaris*, that will allow the analysis of the protein and its purification processes.

Although the work in the environmental and animal fields is more recent and the community is smaller, research is showing encouraging results. Projects in waste water treatment, cattle tuberculosis, brucelosis and leptospirosis have been awarded national prizes for their scientific quality. And the research in some criollo cattle breeds has yielded the first in vitro cattle embryos.

GERARDO MARTINEZ LOPEZ
SUBDIRECTOR OF SCIENTIFIC AND
TECHNOLOGIC DEVELOPMENT PROGRAMS

INTRODUCTION

The definition of a strategic agenda for activities in biotechnology in Colombia requires the knowledge of the dynamics of the research community in universities, research centers and enterprises based on biotechnology.

The Technical Secretariat of the National Biotechnology Program of Colciencias, as a response to the strategies planned by the Program's Council, aiming to set up the capacity for monitoring research activity and as a tool for prospective work in biotechnology. This book is the result of a follow-up program for activity in science and technology, including: the recognition of an infrastructure for research and development in biotechnology; the characterization of the researching community and the valorization of the contribution of research groups, measured in terms of the production of goods, services, publications in national and international journals.

As a result of this effort, today we know about the dynamics of the country's biotechnology community, in terms of research groups, research areas, academic level an areas of researchers, research needs and offer, international and national cooperation links, publications and financed projects, among others.

This is the updated version of "Biotechnology. Five years of research, 1991-1996", with information related to projects supported by the National Biotechnology Program until 1997, to research groups working within universities, research centers and enterprises. It is a part of a series published by the Program, including the following books:

- Tecnologías de la Vida para el Desarrollo, 1993
- Directorio de Biotecnología. Colombia, 1995
- Biotecnología. Cinco años de investigaciones en Colombia, 1991-1996, 1996
- Biotecnología en Colombia. Grupos de investigación, 1998
- Biotechnology in Colombia. Research Groups, 1998

In the same series, there are other titles related to the legal frame of biotechnology, and its application to food science and technology:

- Biotecnología. Legislación y gestión para América Latina y el Caribe, 1994
- Procesamiento y Conservación de Alimentos en América Latina y el Caribe, Volumen I, 1996
- Procesamiento y Conservación de Alimentos en América Latina y el Caribe, Volumen II, 1997

This book compiles the research activity of 71 projects supported by the National Biotechnology Program, from its beginnings in 1991 to 1997. The book is divided in two sections, classified according the application field of the research: agricultural, environmental, industrial, human health and animal. The first section shows the results of 30 projects already finished. Among them, there are 16 projects in agriculture, 6 in industry, and 6 in human health, and additional projects in environmental and animal biotechnology. The last section shows the results of the 41 now in development: 25 in the area of agriculture, 5 in industry, 5 in animal biotechnology, 4 in human health and 2 in environment.

The text of this book will be available from June, 1999, in the homepage of the Colombian node of OAS's Simbiosis (The Biotechnology and Food Technology Specialized Information System): http://www.colciencias.gov.co/simbiosis/

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INDEX

II. ENVIRONMENTAL BIOTECHNOLOGY

III. HEALTH BIOTECHNOLOGY

IV. INDUSTRIAL BIOTECHNOLOGY

V. LIVESTOCK BIOTECHNOLOGY

Section one

COMPLETED PROJECTS

I. AGRICULTURAL BIOTECHNOLOGY

ADAPTATION OF MICROPROPAGATION TECHNIQUES TO THREE FRUIT SPECIES

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OBJECTIVES

- Utilize the new biotechnologies, especially *in vitro* plant tissue culture for the clonal micropropagation of the following three fruit species: Citrus: *Citrus sinensis* var. *Valencia* and var. *Washington*. Passion fruit: *Passiflora edulis* var. Flavicarpa degener and var. Purpura *sims*. Pineapple: *Ananas comosus* var. *Cayena* and var. *Manzana*

- Carry out phytosanitary treatment of the plant materials using meristem isolation and culturing techniques and/or thermotherapy or micrografting in order to provide growers with materials free of the five main viruses that may affect production.

SUMMARY

Fruit growing in Colombia has evolved in the last few years in response to a higher demand for quality by national as well as by international consumers. This has led to an increase in technification of these crops.

One of the unresolved problems is in the production of high quality propagation materials, resistant or tolerant to diseases, as well as in the conservation of germplasm with valuable agronomic characteristics.

Among the most appreciated species in the national and international markets are passion fruit (*Passiflora edulis*) and pineapple (*Ananas comosus*), therefore this project was designed to develop and optimize micropropation techniques for these species.

In the case of passion fruit a high level of genetic diversity (heterozygosity) is observed due to its open pollinization and the traditional propagation system used (sexual). This in addition to the yield loss caused by the presence of viruses creates the necessity to develop techniques that allow mass propagation of clones with desirable agronomic characteristics (productivity, fruit size, resistance to pathogens, adaptation to various stresses, etc).

Within the frame of this project *in vitro* tissue culture techniques for the micropropagation of passion fruit (Passiflora edulis var. *Flavicarpa* and var. Purpura sims) and pineapple (*Ananas comosus* var. *Cayena* and var. *Manzana*) will be used. Phytosanitary cleaning of plant materials will be carried out using meristem culture and/or thermotherapy or micrografting.

In the meantime the methodology for the *in vitro* multiplication of these materials has been implemented.

The best results for passion fruit have been obtained by adding simultaneously the

cytokinins benzylaminopurine (BAP) and kinetin (K). It was determined that BAP at concentrations below 0.5 mg/mL reduces shoot formation during the initiation phase. The best conditions found for shoot induction at nodes were obtained for BAP and K at 0.75-1.5 mg/mL and 1.05-2.1 mg/mL, respectively. Shoot induction with BAP alone is 75% below that obtained with both hormones administered simultaneously.

With pineapple we found that axial buds of the crown and shoots and those from the main stem behave differently with respect to disinfection and during the induction phase. Axillary buds of the stem are difficult to disinfect, probably due to their soil vicinity, which facilitates contamination. Based on the results obtained it can be predicted that starting from 50 to 70 axillary buds ca. 15 million plants can be obtained in 18 months.

From the members of the viral complex present in Colombia in the species *Passiflora edulis* the filamentous and the isometric viruses, members of the potyvirus and the tymovirus families, were studied. The filamentous virus isolated presents a different behavior as that displayed by other known potyviruses; e.g., the PWV (Passion fruit woodiness *Potyvirus*, PWV) causes a malformation of the fruit that has not been observed in Colombia. Inoculation of the virus on *Nicotiana benthamiana* induces the formation of anular spots (strong mosaic) while PFRSV (Passiflora ringspot *Potyvirus*, PFRSV) does not. Furthermore, the potyvirus antiserum utilized to detect PWV as well as PRV does not react with the isolated virus, nor does a CMV (Cucumber mosaic *Cucumovirus*, CMV) antiserum, which suggests that this might be a novel virus.

On the other hand, the symptoms of the tymovirus isolated in Colombia are similar to those described for the PYMV (Passion fruit yellow mosaic *Tymovirus*, PFYMV) isolated in Brazil. Both present a yellow, shiny mosaic and chlorosis in old leaves.

APPLICATION OF THE MOST ADEQUATE BIOTECHNOLOGY FOR THE PRODUCTION OF UNICELLULAR PROTEIN FROM *Spirulina maxima*

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OBJECTIVES

- To establish *Spirulina* sp. populations in the laboratory.
- To standardize different growth media for *Spirulina maxima*, utilizing waste material, especially the effluent of anaerobic biodigestors.
- To implement production systems that allow an efficient agitation of the growth media, especially under field conditions, using eolian, hydric or mechanic approaches.
- Once a constant production of *Spirulina maxima* is established, use it as a proteic supplement for various small animals: ducks, chicken and as a last resort pigs, animals that utilize basic diets with non-conventional energy sources (sugar cane juice, molasses and plantain, among others).

SUMMARY

The microalgae *Spirulina maxima* is an autotroph with a high photosynthetic efficiency, it is being used as protein supplement in the diet of various animals and even for human beings. The definition of growth conditions and the establishment of small production plants may contribute finding solutions to environmental contamination problems, reutilization of water with organic and agroindustrial waste.

In this project we developed a methodology to grow *Spirulina maxima* in effluents of continuous flow biodigestors fed with pig excrements with a retention time of 60 days (1:1 water, effluent) and residual effluents from the liquor industry (1:1 water, effluent) adding 10 g/L and 34 g/L respectively of sodium bicarbonate to raise the pH above 9. We obtained contents of N = 7%, P = 0.2%, K = 0.8% and Ca = 1% on dry base.

Under field conditions we found that the water hyacinth *(Eichornia crassipes)* was more efficient in improving the water quality coming from biodigestors and thus had a positive effect on the productivity of the algae as compared to the species *Lemna sp, Azolla anabaena* and *Pistia stratiotes*.

In the consumption tests of *Spirulina* with fattening ornamental chicken no significant differences were found in the consumption (1.83 g/animal/day), in weight increase

(123 g/animal) and in the percentage of conversion (1.3 %) with the control of soy cake. In the case of young ornamental fish (*Caracius auratus*) fed with *Spirulina* and *Artemia* (1:1) a survival rate of 64 % was obtained vs. the treatment with 100% *Spirulina* which gave a survival rate of 0 %, whereas a survival rate of 77 % was obtained for the control (*Artemia salina*)."Fresh consumption of this cyanobacteria in dancing fish may increase when offering live food, as it was done with *Artemia salina*, while the viviparous fish may turn into better consumers of dry *Spirulina* after 25 days of age."

BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF INDIGENOUS STRAINS OF *Bacillus thuringiensis* FOR PEST CONTROL IN AGRICULTURE

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OBJECTIVES

- Establishment and maintenance of indigenous strains of *Bacillus thuringiensis*.
- Biochemical, molecular and biological characterization of indigenous strains from the *Bt* bank of the (National Program for Integrated Pest Management).
- Selection of *Bt* strains with potential for insect control, of the Lepidoptera and Coleoptera orders.
- Standarization of growth conditions for *Premnotripes vorax*, and perform bioassays with reference *Bt* strains.
- Bioassay evaluation, in laboratory, of pesticide activity of indigenous strains of *Bt* against *Tecia solanivora* larvae.
- Field evaluation of the insecticidal activity efficiency of the seleded *Bt*. strains against *Spodoptera frugiperda*.

SUMMARY

Due to the economical impact of pests such as *S. frugiperda*, *T. solanivora* and *Premnotripes vorax* on some crops, and to the indiscriminate use of chemical pesticides for their control, it is necessary to supply alternatives for biologic control. The main objective of this work is to evaluate and select native strains of *Bacillus thuringiensis* with high pesticidal activity against these pests, taking into account the country's biodiversity and thus, the possibility of finding better adapted microorganisms to tropical conditions than foreign ones.

A bank of sporulated bacili was collected, with 3,052 selected isolations from soil and sick insect samples taken from different regions of the country (Cundinamarca, Boyacá, Valle del Cauca, Nariño, Guajira, Meta, Caquetá, Amazonas, Antioquia and Norte de Santander). 600 out of these samples have been identified as *Bacillus thuringiensis* by the paraesporal crystal. The isolations are preserved in paper slips inside glass blisters, at 4°C, and maintenance, viability and conservation tests have been performed in 600 isolations.

488 isolations have been biochemically characterized, and 309 out of them (63%) presented electrophoresic profiles related to the reported in literature for proteins active Cry1 lepidoptera-diptera (29%), active Cry2Aa diptera (23%), active Cry3Aa coleoptera (7.4%), active Cry1Ia lepidoptera-coleoptera (10%). 107 (19%) out of them presented new profiles, and 72 (14%) did not present a variable profile. 422 isolations have been

molecularly characterized, and 371 (82%) presented amplification, and the gene families active Cry1 lepidoptera (54%), active Cry2 lepidoptera-diptera (25%), active Cry3 coleptera (5%), and active Cry11a lepidoptera-diptera (9%) can be recognized. 17% did not show any amplification.

73 native strains have been evaluated, with a dosis of 200 mg/ml spora-crystal, against first instar *Spodoptera frugiperda* larvae. 4 of these strains have been selected because of their insecticide activity: Bt127, Bt1165, Bt2468 and Bt3107, with mortality rates going from 80% to 90%. The evaluation of possible combinations of 3 out of these strains (Bt127, Bt1165 and Bt3107) allowed the selection of a promissory combination for pest control: Bt1165 and Bt3107, with an average lethal concentration of CL50 88mg/ml of total protein represented by a mortality rate of 87% during 120 hours. This activity was not very different from the reported activity for the HD-137 strain, with a CL50 of 106mg/ml and a mortality rate of 79% during the same time lapse. Against *Tecia solanivora*, 46 strains have been evaluated. Strain Bt2468, from Leticia (Amazonas) and strain Bt3107, from El Espinal (Tolima) were selected by their high insecticide activity. They caused acumulative corrected death rate of 90% and 72% respectively, during 96 hours; while HD-1 strain presented a death rate of 95%, without significative differences to the indigenous strains. The results showed that in different parts of the country there are strains with a high-controlling capacity, although they show the difficulty of finding potential strains as only 4% of the evaluated strains had high activity against this pest.

For *P. vorax* bioassays, minimum breeding conditions were established for this insect. The result showed that the *B. thuringiensis var. tenebrionis* can be used in further evaluations as it produced a death rate of 86.9%.

For the standarization of mass production conditions of indigenous strains, different culture media have been evaluated. The fermentation of the strains was designed, and an optimal substrate was found, composed by: yeast extracts, molasses, organic and inorganic nitrogen and salts. Previous tests have been performed, in experimental plots, with the product Xentary, based on *Bacillus thuringiensis* var Aizaway HD-137, for standarizing and determinating the required conditions to evaluate the indigenous strains efficiency in field. These strains were selected in laboratory for *Spodoptera frugiperda* control. In the future, native isolations of *Bacillus thuringiensis* will continue to be evaluated, as they present a high potential for pest control.

BIOTECHNOLOGY AT THE SERVICE OF THE COLOMBIAN FARMERS: DEVELOPMENT AND PROPAGATION OF RESISTANT VARIETIES TO THE YELLOW AND BLACK SIGATOKA IN PLANTAIN AND BANANA AND DEVELOPMENT OF VIRUS FREE VARIETIES IN PASSION FRUIT AND POTATO

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OBJECTIVES

- To develop and propagate varieties resistant to pests and diseases, especially in asexually propagated crops.
- To produce materials free of viruses and other pathogens, guaranteeing their genetic purity.
- To develop integrated pest and disease management systems by introducing resistant or tolerant varieties and the rational use of pesticides, biological and natural control.
- To offer diagnostic services for pests and diseases using modern and safe techniques that allow adequate, quick and reliable indexation.
- To produce at an industrial level beneficial organisms for pest control as well as for biological nitrogen fixation using *Rhizobium* strains of known efficiency.
- To produce material free of viruses and other pathogens in passion fruit and potato.
- To evaluate and propagate plantain and banana varieties resistant to both Sigatokas available in the germplasm bank of BIOTECOL Ltda., in their respective ecosystems.

SUMMARY

Production of banana and plantain is affected by black Sigatoka (*Mycosphaerella fijiensis*), the bacterial Moko disease (*Pseudomonas solanacearum*) and by the nematode *Radophulus similis*, the production of passion fruit by fungal (*Fusarium*) and viral diseases (*Potyvirus*, *Tymovirus* and *Closterovirus*) and the potato production by various viruses. This is why they require huge amounts of agrichemicals which contaminate the product as well as the environment.

In the course of this project protocols have been developed that allow an increase in the multiplication rate of plantain with the genome AAB and ABB of the Dominico Harton and Harton types as well as for the multiplication of varieties resistant to black Sigatoka and to Panama disease, races 1 and 4, developed by the Honduran Foundation for Agricultural Research (FHIA), with tetraploids genomes AAAB: FHIA-01, FHIA-02, FHIA 03, FHIA 18, FHIA-21 and FHIA-22. Also 50.000 banana and plantain *in vitro* plants were propagated and distributed, corresponding to the varieties FHIA-01, -02 and -3, -21 and -22 and Africa-01 for the black Sigatoka campaign of ICA´s Plant Health Program, as well as 1000 banana *in vitro* plants and 4200 plantain plants for CORPOICA´s Plantain and Banana Program, which are currently being evaluated

agronomically and for resistance to black Sigatoka, pests and other diseases in different experimental centers of CORPOICA all over the country in different ecosystems.

Furthermore, plants of the variety Gros Michel, Valery and Gran Nain, and William have been obtained through micropropagation, marketing has started for these varieties in the coffee zone and in Ecuador.

On the other hand the plantain variety FHIA-21, being commercialized with Ecuador, has been propagated.

ICA has already multiplied the banana FHIA-0 and the plantains FHIA-03 and FHIA-21 to be distributed among the small farmers where S. negra has destroyed their varieties Dominico-Harton and Harton. In this way, they are obtaining again their daily food, and living in a sustainable agriculture and a better environment.

There are commercial, fields of FHIA-21 in areas where S. negra does not permit to grow Dominico-Harton and Harton. This variety has been accepted commercial by the consumers in the cities near to the Magdalena Medium, Arauca, and Caquetá.

Virus-free passion fruit plants (free from potyrirus, the most commonly found of the mosaic group of soybean (SMV), and tymovirus) have been obtained using thermotherapy, and from selected seeds provided by the casa Grajales. For potato we have the technology needed for micropropagating the main varieties *Solanum tuberosum, S. andigenum* and *S. phureja*.

CHARACTERIZATION OF THE PROTEINS AND GENES RESPONSIBLE FOR TOXICITY OF *Bacillus thuringiensis* ssp Medellín

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OBJECTIVES

- To determine the insecticidal characteristics of the toxins of *Bacillus thuringiensis* ssp Medellín. (*Bt.med*)
- To design probes to isolate the gene coding for the 68 KDa protein of *Bt* ssp Medellín based on the aminoterminal sequence of the protein.
- To sequence the genes coding for proteins p94, p68 and cytM1.
- To determine the insecticidal characteristics of mixtures of the proteins from *Bt israeliensis* and *Bt* ssp Medellín.

SUMMARY

An enriched preparation of crystals of *Bacillus thuringiensis* ssp *Medellín* (Btmed) was obtained and solubilized. The solution was fractionated using size exclusion chromatography on Sephacryl-200. Three main peaks were observed in the chromatogram. The first peak contained two proteins that migrated between 90 and 100 KDa. These proteins are apparently uncommon in other *Bacillus thuringiensis* (*Bt*) strains isolated to date. The second and third peaks contained two polypeptides of 68 and 28-30 KDa, respectively. Each peak, independently, showed toxicity against first instar larvae of *Culex quinquefasciatus*. Interestingly, combinations of the fractions corresponding to the 90-100, 68 and 30 KDa proteins showed a slight increase in toxicity. Preliminary results suggest that the 94 KDa protein is an important component of Btmed's toxins, having the lowest mean lethal concentration (LC50). Additional studies will be necessary to completely elucidate the interaction between the crystal proteins. An antiserum against the 90-100 KDa protein showed cross-reactivity against the 68 KDa protein. The antiserum did not react against other *Bt* species on a Western blot, demonstrating that Bt.med's proteins are immunologically different from other species. Two gene libraries were generated using total DNA of Bt.med. The analysis of the libraries has allowed to obtain the genes corresponding to the toxic 94 and 30 KDa proteins. Partial restriction maps of both genes have been elaborated. Sequencing of the genes is in progress, with at least 25% of the genes sequenced by now.

DEVELOPMENT OF A SYSTEM TO PRODUCE TRANSGENIC COFFEE PLANTS

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OBJECTIVES

- To select and evaluate vectors for the introduction of foreign DNA into coffee cells.
- To evaluate the transient expression of reporter genes and select the transformed genes.
- To evaluate the stable expression and integration of the genes in the cells, calli and plants.
- To construct a DNA library of *Coffea arabica* var. *Caturra*.

SUMMARY

With the development of genetic engineering methods during the last decades, many plant species, including mono and dicotiledons, can be transformed genetically with genes isolated from different sources. These methods can be complementary to traditional crossing and selection methods, generating new sources of genetic variability. In the case of coffee, the development of a genetic transformation protocol will allow on one side the availability of a system to incorporate resistance genes against the coffee berry borer (*Hypothenemus hampei)* into the variety Colombia and, on the other hand, it will serve as a tool to conduct basic studies on the function and regulation of genes of importance to the coffee plant.

In the first phase of this study, protoplasts isolated from embryogenic cell suspensions were used to study the transient expression of a reporter gene, the β-glucuronidase gene (*gusA*). Conditions for the introduction of DNA mediated by polyethylene glycol (PEG) and for the transient expression of the *gusA* gene were established. The results obtained during this phase of the study showed that it was possible to obtain high levels of transient *gusA* activity in coffee protoplasts, after application of the appropriate conditions PEG mediated introduction of DNA. Once the adequate conditions for transient gene expression of the *gusA* gene were established, a second phase was initiated to define the conditions for stable protoplasts transformation. As a marker gene we used the neomycin phosphotransferase gene (*neo*), which confers resistance to kanamycin. The obtained results showed that the PEG method could be reproducibly used for the stable transformation of protoplasts of the C. *arabica* genotype 81/4-302 used in this study. Finally, the transgenic character of the recovered calli was demonstrated by evaluating the activity of the enzymes Neomycin Phosphotransferase II (NPT II), and β-Glucuronidase (GUS) and by using the polymerase chain reaction (PCR) and the Southern techniques. During the development of the present study it was not possible to obtain regenerated plants. Therefore, we plan to continue working on this aspect until plant regeneration from the recovered embryogenic calli is achieved, and start new transformation studies with other genotypes. Additionally, a DNA library of *Coffea arabica*

var. *Caturra* was constructed, a protocol for the isolation of genomic DNA was developed, which satisfies purity, size and quantity requirements for the construction of a genomic library. The genomic library was constructed using the phage Lambda GEM-11 as a cloning vector. The titer of the gene library was estimated to be $1.02x10^{10}$ pfu/mg of vector DNA.

Finally, a complementary DNA (cDNA) library was constructed. For the construction of the cDNA library, seeds from the component S34 of the Colombia variety was used, the seeds were collected 30 weeks after flowering (stage susceptible to the borer attack). As part of this work, a method for seed collection in the field, that guarantees total RNA integrity, was developed; a protocol for total RNA extraction and mRNA purification, was adopted; and the synthesis of the cDNA was completed with mRNAs isolated from 30 weeks old seeds. The cDNAs were cloned in the vector lambda gt10. The library titer was estimated to be 5.3XI05pfu/ml (plague forming units per milliliter) that corresponds to a concentration of 1.06X106 recombinants/pg of vector DNA. This titer guarantees the presence of low abundant mRNAs corresponding to low copy or one-copy genes. Presently, a primary screening of the library, with total coffee DNA, is underway.

DEVELOPMENT OF NEW DIAGNOSTIC METHODS FOR PLANT VIROSES

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OBJECTIVES

- To validate by double blind laboratory assays the ELISA techniques for the detection of the Bean southern mosaic *Sobemovirus.semovirus*, SBMV, and the Bean common mosaic *Potyvirus* BCMV.
- To distinguish in one single assay the different strains of the common mosaic virus.
- To validate the DNA probe obtained from the Bean common mosaic virus on field samples.
- To utilize the synthetic probe developed for *potyvirus* on other viruses important in passion fruit.

SUMMARY

The Bean common mosaic *Potyvirus* (BCMV) is a viral complex that causes great productivity losses. Depending on the cultivar, the virus produces a characteristic mosaic while others produce systemic necrosis, and therefore have been considered not to belong to the BCMV complex.

In the course of this project diagnostic methods for the *potyvirus* BCMV were developed, including immunologic methods of the ELISA type based on polyclonal antisera against the viral capsid purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Antisera were prepared against necrotic (NL-3) and non-necrotic (Florida) strains of BCMV, and ELISA tests of the sandwich type were developed to detect viral infection. The sensibility obtained was moderate, as some infected plants presented values below the detection level and the specificity was inadequate as well, mainly due to the presence of false positives in healthy plants. Additionally, polyclonal antibodies were developed against the Southern Mosaic Virus, in this case the ELISA was very sensitive and showed good specificity (0.90).

Based on recombinant DNA technology two methods were developed: 1. A synthetic oligonucleotide probe based on the sequences published for various *potyvirus*. The probe recognizes the *potyvirus* with variable sensitivity and does not react with other plant virus, and 2. A cloned probe obtained using reverse transcription, cloning and transformation. We obtained several clones in the vector Bluescript KKS II (+/-) (Stratagene) that reacted specifically with the viral RNA of NL-3. Using peptide mapping we obtained evidence that indicated that NL-3 is a novel virus. The peptidic pattern obtained with Staphylococcal protease V8 is significantly different from the non-necrotic strains, like the Florida type.

IN VITRO STORAGE OF PASSION FRUIT

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OBJECTIVES

- Development and adaptation of tissue culture techniques for the conservation, maintenance and propagation of *Passiflora* genotypes, in order to provide material for breeding programs important species for agriculture.
- Selection and collection of plant material for *in vitro* storage of Passion Fruit.
- *In vitro* establishment and development of micropropagation and slow growth systems for germplasm conservation.
- Evaluation of genetic stability of the stored material using molecular markers.

SUMMARY

The *Passiflora* genus represents a resource of great potential among tropical fruits. Due to the variety of species adapted to different altitudes and to its suitability for human consumption, its economic importance has increased either at domestic and international markets during the last years. This situation suggests the need for characterizing the germplasm, creating gene banks and developing *in vitro* culture techniques in order to support conservation, propagation and varietal breeding of these species.

In this study, several genotypes of wild and cultivated species belonging to the *Passiflora* genus were recollected and identified. A system for *in vitro* storage under slow growth conditions (10°C and 20°C during 18 and 12 months) was developed for two model species *(Passiflora edulis* var. *flavicarpa* and *P. mollissima)* showing successful regeneration after periods of more than 12 months at minimum growth storage. Molecular marker techniques (restriction fragment polymorphisms of cpDNA and isozymes) were used for the characterization of the collected accessions and screening of genetic stability during *in vitro* handling. The micropropagation assays with other species *(P. mollissima, P. edulis, P. caerulea, P. maliformis, P. erythrophylla, P. cuspidifolia)* has shown a high *in vitro* response of nodal segments.

Additional work was undertaken for the plant regeneration of *P. edulis* var. *flavicarpa* (passion fruit) and *P. mollissima* (banana passion fruit) through organogenesis from leaf discs. Passion fruit leaves were cultured in Murashige & Skoog basal medium with different concentrations and combinations of growth regulators, the best induction and shoot development results were obtained from a combination of N6-benzylaminopurine (4.4 -13.3 mM) and kinetin (2.3 - 4.6 mM). Elongation and rooting of the shoots were achieved in MS basal medium without growth regulators. In the same way, an equally reliable system for the regeneration of banana passion fruit plants from leaf explants from *in vitro* cultivated nodal segments was developed. Shoot regeneration was

obtained using BA (13.3 mM) and kinetin (9.3 mM) in a modified Nitsch medium. The development and rooting was held in the same medium without growth regulators. The plant regeneration systems developed for these two species (of major economic importance) represent an important progress for further genetic manipulation and genetic transformation studies in *Passiflora* species.

In the molecular markers analysis, clearly discrete restriction patterns were obtained with Xho I, Dra II, Bam HI. Hind III and to a less degree by Bgl II, Dra 1, Eco RI, Hae III, Sac I and Sma I, whereas Kpn I, Mbo I and Pst I showed diffuse patterns. Polymorphisms were useful to differentiate between species, thus confirming the potential of restriction fragment analysis in molecular characterization studies. Amplification techniques for non-coding cpDNA sequences using total DNA were also optimized. The higher mutation frequency of non-coding regions was useful for genotype characterization and evaluation of genetic stability. The results obtained so far have not shown that the systems developed for micropropagation and storage may induce variations in the material and thus do not represent a risk to preserve the genetic uniformity of the accessions.

After the three-year research, around 91 accessions were localized and collected. In addition to the development of *in vitro* storage techniques for "banana passion fruit and "passion fruit", methods for *in vitro* management of commercial (*P. edulis* var. flavicarpa, *P. ligularis, P. maliformis P. mullissima, P. popenovi* y *P. quadrangularis)* and wild (*P. adenopoda, P. caerulea, P cuspidifolia y P. erythrophylla)* species were improved. These methodologies could be used as the basis for the management of the genetic resources of other species.

The study of DNA polymorphic restriction fragments of organelles allowed to design useful systems for germplasm characterization, that could be applied in the study of the genetic relation of other species with unknown genomes. The cloning of specific sequences was also initiated, so that they could be used in the future as probes for germplasm characterization.

ISOLATION AND CHARACTERIZATION OF PLASMID DNA OF BACTERIA USEFUL FOR BIOLOGICAL CONTROL

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OBJECTIVES

- Isolation, identification and selection of native bacteria from different regions of the country used in biological control.
- Isolation and amplification of toxigenic plasmids from native bacteria used for biological control.
- Isolation and characterization of plasmid DNA from isolated native bacteria: *Bacillus sphaericus, B. thuringiensis.*
- Cloning and amplification of DNA toxigenic plasmids and/or chromosomal genes from *B. cereus* and *E. coli.*

SUMMARY

Since the introduction of the term "biological control", spore-forming bacteria have been studied extensively. The possibility of obtaining toxic products during the process of sporogenesis have made them a fundamental alternative to chemical pesticides, fungicides, etc., for the control of insect pests or disease vectors in animals and plants.

Genetic studies of chromosomal and plasmidic DNA have established a relationship between the presence of the plasmid and /or chromosomal genes and the production of toxic proteins (e.g., toxin) in *B. thuringiensis* and related organisms, as well as in *B. sphaericus.*

In this project we isolated and identified 278 strains of spore-forming bacilli from samples taken from water, soil, larvae and leaves collected in different regions of the country (Llanos Orientales, Atlantic Coast, Coffe-growing zone, Pacific Coast). Identification was based on the taxonomic criteria cited in Bergey´s Manual and by comparison to control strains directly obtained from ATCC or supplied by other research groups. The following strains were employed: for *B. thuringiensis* strains DH, DH 2, IPS82 and H14; for *B. sphaericus*, strain 2362.

To evaluate the toxicity of the selected strains, bioassays on third instar larvae of *Culex quinquefasciatus, Anopheles albimanus* and *Aedes aegypti* were carried out. Twenty-two strains showed a mortality rates of 80-100% between 24 and 72 hours after infection. Of those 19 were classified as *Bacillus sphaericus* and 3 as *Bacillus thuringiensis.* The 22 strains had high molecular weight plasmids; to correlate their presence with toxicity, bacteria were cured by heat shock and acridine orange. Four native strains showed plasmids associated with their toxicity, and it was demonstrated through transformation in one strain that stability of those plasmids can vary. It is important to underline that these strains represent a promising alternative for biological control and that they possess plasmids of larvicidal activity that offer an advantage in relation to recycling and dissemination of the microorganism.

ISOLATION OF *Bacillus thuringiensis* STRAINS ACTIVE AGAINST COLEOPTERA USING PCR WITH CRY GENES SPECIFIC PRIMERS

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OBJECTIVES

- To isolate *Bacillus thuringiensis* and *Bacillus sphaericus* strains from different soil types of the savanna of Bogota with insecticidal activity against larval stadiums of coleoptera and characterize these strains using molecular biology techniques.
- To isolate *B. thuringiensis* and *B. sphaericus* strains from soil and infected insect cadavers found in agricultural fields and pasture land from different areas of the savanna of Bogota.
- To characterize *B. thuringiensis* and *B. sphaericus* strains with coleoptera larval activity using PCR with primers specific for the CryIIIA, CryIIIB, CryIIIC, CryIIID, CryV and CryC genes.
- To establish a gene and a strain bank of *B. thuringiensis* and *B. sphaericus* strains isolated from different soils around the savanna of Bogota.
- Carry out preliminary *in vivo* assays under laboratory conditions.

SUMMARY

Bacillus thuringiensis strains showing activity against grubs, the larvae of coleoptera of the *Scarabaideae* family, have been isolated from soil samples and from intestinal tissues of infected insects gathered in agriculturally important areas in Colombia. The isolated strains were screened applying a PCR assay using specific primers for the different Cry genes that have been reported in the past to act against coleoptera. Several strains showing positive amplifications were then tested *in vivo*, showing variable activity against grubs. Strain 07-03, isolated from the intestines of a dead insect found in an infested pasture, showed positive results after amplification with primers specific for CryIIIC genes and it showed further insecticidal activity against second instar larvae of grubs of the genus *Clavipalpus*.

Given the fact that at least six different genes have been described in the past as being active against coleopteran larvae, and because we wish to carry out the least possible number of PCR assays, we decided to construct a primer that amplified the largest number of genes in one single reaction. Analysis using FASTA (Genetics Computer Group, Madison, WI) were made using the 22 gene sequences found in the CD-ROM Entrez database (NCBI, Bethesda, MD) corresponding to the genes CryIII and CryV. It was concluded from this analysis that the sequences of the genes CryIIA, CryIIIB, CryIIIB2 and CryIIID showed enough homology at their 5'ends that a set of primers (MP 1 and MP 2) were sufficient to amplify all four genes. The genes CryIIIC and CryV are different, therefore primers are needed, that amplify them separately (MP 11 and MP 12 for the CryIIIC gene, and MP 19 and MP 20 for the CryV gene).

Each one of the hundreds of *B. thuringiensis* strains isolated during the course of this study was subjected to this test using three PCR reactions: the first with the primers MP 1 and MP 2, the second utilizing the primers MP 11 and MP 12, and the third with primers MP 19 and MP 20.

MOLECULAR BIOLOGY OF THE CITRIC TRISTEZA VIRUS: ISOLATION, PURIFICATION AND MOLECULAR CHARACTERIZATION OF VARIOUS MILD AND SEVERE VIRAL STRAINS AFFECTING CITRUS FRUITS IN COLOMBIA.

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OBJECTIVES

- To develop a methodology to diagnose and study the Citric Tristeza Virus (Citrus Tristeza *Closterovirus*, CTV) at the molecular level starting from different strains affecting the productivity of the most widely used varieties obtained from five specific regions in Colombia.
- To purify the different tristeza virus strains present in Colombia.
- To define the RNA and the viral protein of the different strains with their physicobiological integrity.
- To characterize the RNAs and the structural proteins based on molecular weight determination using polyacrylamide gel electrophoresis.
- To establish the genetic relationship between the different viral strains through molecular hybridization of their genomes.
- To produce various molecular probes by synthesizing the respective cDNAs, cloning and amplification in the *E. coli* plasmid pBR322.
- To produce polyclonal and monoclonal antibodies against different CTV strains found.
- To establish the ELISA methodology to produce the diagnostic kits.
- To sequence the genome of at least one mild and one severe CTV strain in Colombia.

SUMMARY

The work presented here describes the results of an epidemiological and molecular survey about the situation of the Citric Tristeza in Colombia that took place between the years 1990 and 1993. The presence of the CTV (Citrus Tristeza *Closterovirus*, CTV) was assessed on the basis of the reaction of citric tissue extracts in the ELISA assay using polyclonal and monoclonal antibodies. By means of this assay it was determined that 95% of the citrus trees were actually infected with severe CTV strains except those from the Mompox region, where 70% of the productive citrus trees were infected only with the mild CTV strains. The immunoblotting analysis showed that the serologic reaction of the infected tissue extracts was due to the presence of proteins with the same molecular weight as the protein of the capsid (CP) of CTV. The electrophoretic study of the dsRNAs isolated from the infected tissues confirmed the presence of CTV, apart from showing the complexity and diversity of the dsRNA profile. These results suggest the association of various viral strains in one tree, which constitutes a severe menace to Colombian citrus culture due to the possibility to create new and more aggressive viral associations through dissemination by the vector *Toxoptera citricidus*. Sequencing the gene coding for the protein of the capsid of CTV

(GCP) confirmed the serologic classification of the CTV as mild and severe strains and contributed to define the evolutive molecular pattern of this gene in relation to reported sequences of CTV strains in other countries. Restriction analysis of the GCP sequences defined the presence of an EcoRI site characteristic of the severe CTV strains in Colombia. The knowledge of the GCP sequences led to the design of molecular probes and specific oligonucleotides for the diagnosis, detection and identification of Colombian CTV strains. Serologic data of the dsRNA and the DNA sequence of the GCP suggest that the mild CTV strains of the Mompox region may act as potentially protective agents in conventional cross protection tests and also for the genetic transformation of citric plants to make them resistant to CTV infection.

MOLECULAR CHARACTERIZATION OF CRY GENES AND BIOLOGICAL EFFECTS OF THE DELTA ENDOTOXIN FROM STRAINS OF *Bacillus thuringiensis* (BERLINER) COLLECTED AT DIFFERENT SITES IN COLOMBIA

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OBJECTIVES

- To collect native strains of *Bacillus thuringiensis* at different sites of the country.
- To determine the toxic activity of the delta toxin present in the *Bacillus thuringiensis* strains against lepidopteran and dipteran larvae using biochemical and genetic methods.
- To collect soil samples from different ecosystems of the country.
- To select *Bacillus thuringiensis* strains from the soil samples.
- To characterize electrophoretically the proteins associated with the crystal for each isolated strain.
- To amplify the DNA segments corresponding to genes CryI and CryIV present on the strains using PCR.
- To conduct bioassays to determine the bioinsecticidal activity of the active product of the various strains on dipteran and lepidopteran larvae.

SUMMARY

The purpose of this project is to isolate new Colombian varieties of *Bacillus thuringiensis* (Bt) active against lepidoptera and characterize them using ELISA, SDS-PAGE, PCR and bioassays.

Eighty-nine soil samples from 10 agricultural regions of Colombia were collected for the isolation of *Bacillus thuringiensis* (*Bt*) strains containing gene CryI. A total of 87 samples from the regions of Antioquia, Bolivar, Caldas, Cesar, Cordoba, Cundinamarca, Huila, Tolima and Valle del Cauca were evaluated. From the samples collected by the IBUN (Instituto de Biotecnología de la Universidad Nacional) 1149 spore-forming isolates were obtained, from those 43% (494 isolates) were Bt, which were then submitted to screening for gene CryI. From the samples evaluated by CIB, 1724 spore-forming isolates were obtained, 14.4% (249) of which were *Bacillus thuringiensis*.

The spore crystal preparations of the *Bt* isolates were examined for CryI using electrophoresis (SDS-PAGE). Of the selected isolates 61% showed typical electrophoretic patterns with 130-140 KDa and/or 60-70 KDa bands, other isolates presented prominent bands of 100, 60, 40 and 20 KDa. The electrophoretic profiles obtained were

probed with antisera prepared against the crystal protein *Bt* HD1 var *kurstaki* using immunoblotting techniques. Similarly, spore crystal preparationswere analysed using ELISA tests with antibodies against crystal proteins CryI and CryIII (*Bt* HD1 var *kurstaki* and *BtsI*, respectively) of standard strains.

175 *B. thuringiensis* strains that were pathogenic to *Culex quinquefasciatus* larvae were analysed by SDS-PAGE to determine their protein composition comparing them to standard strains *B. thuringiensis* ssp *israeliensis* (1884) and *B. thuringiensis* ssp *medellin* (163-131). 76% (134) of the isolates showed a pattern similar to *B. thuringiensis* ssp *israeliensis* (1884), with bands of 135, 125-18, 68 and 28 KDa. The remaining 24% (41) showed distinct patterns that deserve a more detailed study to determine their specific characteristics and their potential as biological control agents. These strains were isolated from soil samples coming from the regions of Cundinamarca, Tolima, Huila, Guajira and Cordoba. Additionally, the 28 selected strains were screened using Western blot analysis to characterize their expressed products. *B. thuringiensis* (*Bt*) strains were screened using antibodies against p30, p68 and p94 of *Bt* ssp *medellin* (Btmed). The following observations were made: first, the presence of proteins 30, 68 and 94 KDa characteristic of Btmed toxins, and second, that the migration profile could be assigned to the types *Bt* var *israeliensis*, *Btmed* or and atypic migration pattern that showed cross-reactivity with *Btmed* proteins.

Further analysis was performed on isolates with CryI genes. Using specific 'forward' and 'reverse' primers nine genes were amplified (CryIA(a), CryIA(b), CryIA(c), CryIB, CryIC, CryID, CryIE, CryIF and CryIG).

For this purpose three sets of primers were used (Ceron *et al.*, 1994). Ten different patterns for the Cry genes present in the examined *Bt* strains were found by PCR. 95% of the isolates contained more than one type of Cry genes. Two isolates contained PCR products different from the reference products, suggesting novel genes not described previously and therefore deserving a systematic study of their biological activity.

Twenty-eight isolates were selected at random from the strains obtained and their genes were further characterized by PCR using specific primers for genes Cry1, Cry3 and Cry4 (Carozzi *et al.*, 1991). In 23 of the isolates the presence of the diptera-specific gene Cry4 (A or B) is reported, the lepidoptera-specific gene Cry1A is reported in 4 isolates as well as the absence of the coleoptera-specific gene Cry3.

Bioassays of the CryI gene products were evaluated using *Spodoptera frugiperda* and *Heliothis virescens* as target insects.

The bioassays were conducted on first instar larvae of *S. frugiperda* and *H. virescens*.

Mortality was determined after 120 hours and analysed using Probit analysis. Strains with genes CryIB and CryIE showed the highest toxicity against *S. frugiperda* while CryIF-containing strains showed highest activity against *H. virescens*. It is important to stress that some native strains showed higher toxicity against *S. frugiperda* than standard strain HD1. In later experiments against *H. virescens* it was found that strain IBUN 6.4 is 13% more active than standard strain HD1.

Another set of bioassays was conducted using *Culex quinquefasciatus* as target insect.

First instar larvae were used for this purpose and mortality was determined 72 hours after infection. Out of 150 strains evaluated six isolates showed a toxic activity equal to

the positive control employed in the bioassay (*Bt* ssp *kurstaki*). Two out the six (172-0451 and 172-0761) were included in the PCR analysis and showed the expected amplification products for Cryl.

The pathogenicity range has been determined only for 40 out of 175 diptera pathogens; 31 had a pathogenic range similar to that of *B. thuringiensis* ssp *medellin* (162-131) with a mortality of 100% at 10^{-4} and 10^{-5} dilutions, and 0% mortality in higher dilutions.

Strains 22-09, 24-04, 82-113 and 82-1121, coming from the regions of Bolivar and Cordoba, respectively, had a slightly different pattern, with 100% mortality at a dilution of 10^{-4}, and 80-90% at 10^{-5}, 0% mortality at higher dilutions.

Strain 22-06 from the region of Bolivar showed 100% mortality at a dilution of 10^{-4} and 62% at 10^{-5}, 0% mortality at higher dilutions.

With strains 82-1202, 22-03 and 29-50 from the regions of Cordoba and Bolivar 100% mortality was obtained only at a dilution of 10^{-4}, further dilution led to 0% mortality. Standard strains used were *B. thuringiensis* ssp *medellin* (163-131) and *B. thuringiensis* ssp *israeliensis* (1884).

OBTENTION OF TRANSGENIC POTATO PLANTS RESISTANT TO THE POTATO VIRUS X AND TO THE POTATO LEAF ROLL VIRUS BY TRANSFORMATION WITH *Agrobacterium tumefaciens*

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OBJECTIVES

- To sequence the gene that codifies for the capsid protein of the potato virus (GCP), isolated in Colombia and in France.
- To compare the sequences of the GCP isolates from Colombia, France and other countries.
- To select the capsid gene sequences from French and Colombian isolates potentially effective to provide protection against viral infection in transgenic potato plants.
- To obtain sequence information of the gene coding for the capsid protein (GCP) of the potato virus isolated in Colombia and France.

SUMMARY

The co-infection of potato plants with PVX (Potato X *Potexovirus*, PVX) and PLRV (Potato Leafroll *Luteovirus*, PLRV) reduce tuber productivity to levels oscillating between 65 and 100%. Chemical control of the vector of virus PLRV has been inefficient and ecologically not advisable, while mechanical dissemination of PVX takes place very easily there is no vector known that could be controlled through agrochemical management.

This purpose of this project is to establish methodologies for micropropagation, transformation, regeneration and genetic evaluation of transgenic potato plants resistant to infections by the viruses PVX and PLRV.

More systematic experiments were developed in the model system *N. tabacum* in order to have a positive control or reference system in the transformation and regeneration protocols, and in the biochemical and molecular biology tests involved in the production and characterization of transgenic plants.

The selection of gene sequences of the potato leaf roll virus (PLRV) was achieved. The capsid (CP) protein gene of PLRV was selected from the gene library of PLRV. This library contains the genes of the isolates from the potato producing countries of Latin America; it is deposited in the Scottish Crop Research Institute -SCRI- which is a collaborator in this project.

Furthermore, the CP gene sequence of PLRV was ligated into the vector pSCR107 and introduced into the *Agrobacterium tumefaciens* strain LAV4404. The transformation was performed by co-cultivation of leaf and stem explants of *Solanum phureja* (criollo potato) with the *A. tumefaciens*.

Finally, transgenic *S. phureja* plants expressing the capsid gene of the potato leaf roll virus (CP of PLRV) were regenerated. These *S. phureja* plants are in the process of tuberization and challenge with PLRV strains under glasshouse conditions.

PRODUCTION OF BACTERIAL BIOINSECTICIDES AGAINST INSECT PESTS OF COTTON AND OTHER ECONOMICALLY IMPORTANT CROPS

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OBJECTIVES

- To evaluate the bioinsecticide utilizing laboratory, glasshouse and field bioassays.
- To investigate the separation and purification processes of the delta-endotoxine.
- To study the relationship between the agroecological conditions of cotton cultivation and the efficicacy of the bioinsecticide.

SUMMARY

The agricultural sector has been very important for the economic development of the country; various factors have contributed to the reduction in the market share of crops like cotton, amongst them the attack of insects like *Heliotis virescens* (F.), *Heliotis zea* (Boddie), and *Spodoptera frugiperda* (J. E. Smith). Chemical insecticides have been used to control larvae of these insects, thereby causing contamination problems and showing residual effects that induce resistance in these pests. Biological control employing *Bacillus thuringiensis (Bt)* came up as an important alternative because of the high specificity, biodegradability and its non-toxicity for humans and beneficial animals.

An important component of the technology associated with biological control using *Bt* is the production of the active ingredient by fermentation (entomocidal crystal and spores). In this project the production of *Bt* var. *kurstaki* (HD1) was studied in bioreactors of up to 7 liters, the growth kinetics of the culture were determined, as well as the importance and role of the supllied nutrients in the culture medium. The influence of aereation and agitation on the oxygen transfer coefficient (K_La) considered to be a scale-up criterium in this case was evaluated. The entomocidal activity of different *Bt* isolates was evaluated through bioassays, the life cycle was determined, and artificial diets for *H. virescens* (F.) and *S. frugiperda* (J.E. Smith) were evaluated.

The growth kinetics of the native strains (IBUN7.1, IBUN7.2, IBUN9.1, and IBUN1.6) were established and compared to the standard strain HD1; the overall kinetic performance is similar for all strains and the specific growth rates range from 0.39 h^{-1} to 0.50 h^{-1}. With respect to biomass production it was found that there was no direct relationship with the spore and protein production. In the evaluation of nutrients effects it was found that glucose as a carbon source has a significant effect on the production of biomass and on sporulation. A positive relationship was found between the supply with

yeast extract and glucose, manganese and magnesium sulfate, on the production of biomass, spores and crystal protein.

Using the bioassays three isolates (IBUN6.4, IBUN3.3 and IBUN2.6) were identified as promising for their larvicidal activity against *H. virescens*, and two (IBUN4.0 and IBUN3.8) against *S. frugiperda*.

SELECTION AND PROPAGATION OF *Cordia alliodora*, *Alnus acuminata* AND *Rubus glaucus* BY *IN VITRO* TISSUE CULTURE

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OBJECTIVES

- Selection of basic material of *Cordia alliodora* and implementation of the methodologies needed for mass propagation by *in vitro* plant tissue culture.
- Selection of basic material of *Rubus glaucus* and implementation of methodologies for mass propagation by *in vitro* plant tissue culture.
- Adaptation of methodologies for *Alnus acuminata* micropropagation by *in vitro* plant tissue culture.
- Evaluation of *in vitro* performance and donor tree age dependence of various explants of *Cordia alliodora*, such as leaf buds of adult trees, leaf buds of greenhouse trees and leaf buds of recently germinated plantlets.
- Establishment of the adequate conditions for superficial disinfection of *Cordia alliodora* explants.
- Determination of the adequate culture conditions for each one of the *Cordia alliodora* explants.
- Initiation of a study to establish the best conditions for the adaptation of the micropropagated material of *Cordia alliodora* under greenhouse conditions.
- Selection of young material of *Cordia alliodora* trees in the field that show desirable genetic characteristics, to be propagated and established *in vitro*.
- Determination of the adequate conditions for *Rubus glaucus* mass propagation *in vitro* from selected plants.
- Determination of the best conditions to adapt the micropropagated material to greenhouse conditions.
- Definition of the adequate conditions for micropropagation of selected Alnus acuminata.
- Definition the adequate conditions for the adaptation of *in vitro* produced Alnus acuminata under greenhouse conditions.

SUMMARY

In the Colombian coffee growing region, the change from traditional coffee growing (in the shade) to technified cultivation (without shade) and high-density planting (10.000 plants /ha) has implicated the elimination of a tree stratum of an agroforestal system created by the coffee growers. The objective of this project is the promotion of the cultivation of *Cordia alliodora*, *Rubus glaucus* and *Alnus acuminata* as an alternative source of income for the coffee growers and for promoting the use of native woody species for the protect the watersheds.

Basic plant material was selected from *Cordia alliodora, Rubus glaucus* (var. Castilla) and *Alnus acuminata*. The methodologies for mass propagation using *in vitro* plant tissue culture are being implemented.

Cordia alliodora

For this study, 11 high quality trees (plus trees) were selected in collaboration with the Regional Autonomous Corporation of Risaralda (CARDER) in the municipalities of Marsella, Santuario, Santa Rosa de Cabal, Belén de Umbría and Ulloa.

In vitro culture experiments using leaf buds and node explants of adult trees were undertaken, using field-collected explants, but the high contamination and phenolization levels prevented us from continue using this route. As an alternative we developed a technique of grafting adult leaf buds onto nursery trees, which has allowed us to have leaf buds and shoots from the rejuvenated trees near to the laboratory.

Nursery trees are subject of treatments with fungicides and severe pruning. After that, nodal segments are collected and disinfected with 1% sodium hypochlorite, and washed three times with sterile distilled water.

The following growth media were evaluated: Murashige/Skoog (MS), MS /2 (MS at half concentration), and Woody Plant Medium (WPM). We also tried different concentrations growth regulator. The best response in nodal segments and apical buds was obtained using MS/2 medium and a combination of 1 mg/L kinetin and 0.25 mg/L gibberellic acid. Under these conditions, rapid shooting and stem elongation with concomitant leaf formation was observed.

Seeds were disinfected with 1% sodium hypochlorite during 20 minutes, the embryos were extracted and set on MS/2 medium with 1 mg/L benzyladenine and 0.25 mg/L indolebutyric acid. Once plantlets germinated, cotyledonary and normal leaf segments were excised and set on callus inducing media; different growth regulator concentrations and combinations were evaluated. On MS/2 medium supplemented with 1 mg/L benzyladenine and 0.5 mg/L 2,4D and also on MS/2 supplemented with 1 mg/L 2,4D we obtained a friable, creamy colored callus. Further combinations will be tried seeking to regenerate full plants.

Alnus acuminata

We obtained in vitro regeneration of *Alnus acuminata* H.B.K. Callus was obtained in MS/2 medium with 100 mg/l M-inositol, 3.0 g/l Phytagel and 3,2 mg/l Benzyladenine, 1 mg/l Naftalenic Acid as grouth regulators.

Embryos developed in high rate in MS/2 medium without growth regulators and germination was due in the same medium with O.5 mg/l GA_3.

Plants developed in a medium with 0.5mg/l Indolbutiric Acid and O.25 GA_3. Plantelets are transferred to green house for hardening, they are planted in "forestry pellets" and kept in humid chamber for 10 days. We propose somatic embriogenesis as a way for micropropagation of this species.

Rubus glaucus

We developed a mass propagation system for *Rubus glaucus*, greenhouse hardening and the establishment of plantations on high mountain regions of the Colombian Andes.

For the in vitro mass propagation we used MS/2 medium supplemented with 100 mg/l M-inositol, 0.01 mg/l Tiamina; 1 mg/l Bencil Adenine and 1 mg/l GA_3 as grouth regulators, 2.7 g/l Phytagel.

The substrate for the greenhouse hardening were "forestry pellets", plantelets are kept in humed chamber for five days.

We established a cooperative work with Corpoica and Comité Departamental de Cafeteros for the in vitro production of 100,000 plants during 1998. Up to date we have produced 20,000, that have been planted in Santa Rosa, Santuario and Apia in Risaralda.

STUDY OF THE CASSAVA GENOME BY RFLP AND RAPD ANALYSIS

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OBJECTIVES

- To implement the appropriate techniques to obtain and characterize genomic fragments as markers and arrange these markers into linkage groups.
- To construct a cassava gene library.
- To detect polymorphisms among the fragments of the parental lines.
- To evaluate the segregation of markers in the F_1.

SUMMARY

Very little is known about the genetics of cassava although it is a very important crop in developing countries, like in Africa, Asia and Latin America; the present study offers a starting point for the study of genes associated with important agronomic characteristics, breeding of varieties, new possibilities for the design of crosses, among other possibilities.

The type of inheritance of RAPD (Random Amplified Polymorphic DNA) molecular markers, were studied in an F1 progeny stemming from an intraspecific cross of heterozygote parentals. The analysis confirmed the utility of RAPD markers for the comparison of ideal candidate parentals in order to develop a genetic linkage map and it produced a sufficient number of markers to accomplish a linkage analysis in cassava , a crop with very few genetic studies, classical as well as molecular. Six lines were evaluated as potential parentals for crosses showing between 1.82 to 8.62 segregating bands per RAPD primer in the three hybrid families evaluated. Forty-three percent (309) of the 722 primers produced polymorphic products in the most useful of the three crosses, generating 328 single dose markers which segregated 1:1 (presence: absence) in a progeny of 98 individuals. A second group of markers were those being present in both parents, not polymorphic, but which segregated in the progeny. Fifty-seven (57%) of those markers segregated in a proportion 3:1, an expected segregation in a cross between heterozygotes with single dose markers. Linkage groups with markers coming from each one of the parental lines have been constructed.

II. ENVIRONMENTAL BIOTECHNOLOGY

EVALUATION OF MICROALGAE IN BIOREACTORS FOR THE TERTIARY TREATMENT OF WASTE WATER FROM AGROINDUSTRIAL EFFLUENTS

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OBJECTIVES

- Evaluation of the utilization of algae species isolated from a system of effluent oxidation lakes for the stabilization of organic matter and remotion of nutrients from agroindustrial effluents, as tertiary treatment in a bioreactor.
- Determination of the percentage of nutrient remotion from agroindustrial waste water by microalgal growth in a bioreactor.
- Quantification of the algae biomass production obtained in a bioreactor fed with agroindustrial waste water.
- Evaluation of the reduction of organic matter through bacterial oxidation of agroindustrial waste water.
- Determination of the reduction of coliform bacteria in waste water subject to the tertiary treatment.
- Establishment a strain bank of microalgae for future studies.
- Evaluation of the technical-economical feasibility of a microalgal system as tertiary water treatment.

SUMMARY

Microalgal cultures are an efficient method for the production of biomass and the utilization of nutrients present in waste water and constitue therefore a promising alternative for the production of biomass and agroindustrial waste water treatment.

In this project we compared the growth of the green algae *Chlorella vulgaris* Bejerinck and *Scenedesmus dimorphus* (Turp.) Kütz, isolated from a waste water stabilization pond. Two sterile synthetic media were used to mantain the cultures, C30 and Bold's Basal medium. Additionally, agroindustrial waste water was used as culture medium. Batch cultures were made using both species in 4L cilindrical glass blorreactors (2L working volume). *C. Vulgaris* was also cultivated in a triangular biorreactor. All the cultures were incubated at environment temperature (20 °C), under continuous ilumination (60 micromole/m^2/s) and bubble-aerated. Under these conditions, *C. vulgaris* grew faster and reached a higher call density the *S. dimorphus*.

In waste water with an average content of 8 ppm ammonium, 4 ppm nitrates, 52 ppm total phosphorus, 173 ppm DQO y 49 ppm total suspended solids, the efficiency of *C. vulgaris* in the removal was of 93% for ammonium, 59% for phosphorus, 57% for total

suspended solids and 52% for DQO in the cilindrical bioreactors, and 97% for ammonium, 9% for phosphorus, 72% for total suspended solids and 15% for DQO in the triangular bioreactor. With *S. dimorphus* we obtained a removal efficiency of 92% for ammonium, 33% for phosphorus, 66% for total suspended solids and 1% for DQO.

This study shows the potential of using these microalgae to reduce the environmental pollution of agroindustrial wastewater.

III. HEALTH BIOTECHNOLOGY

BIOCHEMICAL CHARACTERIZATION OF HUMAN HYPOPHISARY HORMONES

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OBJECTIVES

- To obtain pure preparations of some peptidic hormones starting from human hypophisis to be used as standards and for the production of specific antibodies for the production of diagnostic kits in Colombia.

- To study the plasma forms of some peptidic hormones in healthy and pathologic individuals using biochemical and immunological techniques.

- To study the interaction of these hormones with specific receptors present in target cells as a basis for a better understanding of molecular signaling and the effect of pathologic or altered metabolic conditions at the level of protein synthesis and gene expression.

SUMMARY

The advances in modern endocrinology has been mostly due to a better understanding of the biochemical knowledge of the different hormones, their chemical structures, their biosynthetic mechanisms, secretion and targets, and their mechanisms of action.

The present project has been designed to contribute to a better understanding of the molecular forms of the hormones sythesized by the human hypophisis and its secretion patterns, in healthy and pathologic individuals of the Colombian population. The specific interaction with targee cells will allow to develop models that explain the molecular basis of their mechanisms of action and the routes employed in signal transduction, an area where little is known until now.

We developed a methodology for the extraction of the following five hormones from human hypophisary tissue: prolactin (PRL), growth hormone (GH), follicle stimulating hormone (FSH), luteinizing hormone (LH), and tyroid stimulating hormone (TSH), based on their solubility and ionic properties.

Recovery and preservation of activity were determined according to international standards established by the US National Institute of Health (NIH). The contamination level of the preparations was below the one internationally accepted for this type of hormonal standards.

The immunologic potency of the preparations was the following: 10 IU/mg of protein for PRL; 2.7 IU/mg for GH; 1.365 IU/mg for FSH; 28.169 IU/mg for LH; and finally 3.187 IU/mg for TSH. These values allowed us to confirm the high purity achieved in

every single case. The purified hormones were used as antigens for the production of polyclonal antibodies to be used for radioimmune assay (RIA) of each of these peptides and their validation was conducted by comparing to reference antibodies provided by the US NIH.

We conducted some studies concerning the circulating hormones PRL and GH in individuals with metabolic alterations and/or pathological disorders of the hypophisis. The analysis of sera with hyperprolactinemia showed the presence of glycosylated, high molecular weight forms, not detectable in healthy sera, and in the case of acromegaly we found an overproduction of the three species of GH (22K, 45K and 80K), but no forms additional to those found in healthy human sera were found.

Finally, the studies on the interaction with receptors using rats as experimental animals, showed a wide range of somatic distribution for GH. The most relevant result was the identification of specific attachment sites for GH in lymphatic tissue, supporting the recent finding of bi-directional communication between the endocrine and the immune systems. In particular, it was shown that nutritional changes have an effect on the level of expression of the GH receptor in lymphocytes, the spleen and the peripheric blood system. This could have direct implications for humans, because the analysis of GH receptor in peripheric blood, which is easily accesible, growth retardment associated with alterations in the response to GH could be studied. Similarly, this model could be also used to study the relationship between malnutrition and infection processes.

COMMERCIAL PRODUCTION OF REAGENTS FOR HAEMOCLASSIFICATION BASED ON THE GENERATION AND USE OF MONOCLONAL ANTIBODIES TO BLOOD GROUPS A AND B

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OBJECTIVES

- To develop a prototype for the standardization of production methods and industrial scaling up of monoclonal antibodies against various antigens relevant to diagnostics in human medicine.
- To develop production technology and industrial scaling up of monoclonal antibodies than can be important in the study and detection of antigens relevant to diagnostics in human medicine.
- To obtain monoclonal antibodies that will allow haemoclassification utilizing agglutination techniques at least for the major groups A, B, O.
- To develop a prototype laboratory assay that utilizes these antibodies as a basis for A, B, O blood typing.
- To develop a prototype of a commercial kit that will allow to offer this product to the market and to define the pre-scale up conditions in terms of units that will satisfy and/or conquer the national market in the long run.

SUMMARY

Haemoclassification assays are widely used at the clinical laboratory level and in blood banks. Fore the detection of ABO antigens a simple, quick and sensitive agglutination assay is used, in which the red blood cells to be analysed are put in contact with specific antibodies specific for the proteins A or B.

The worldwide trend nowadays is the production of typifier monoclonal antisera against the ABO system. Using this technology a specific antibody to these proteins is obtained with the required specificity and sensitivity characteristics to perform a trustworthy and reproducible haemoclassification, additionally avoiding the biological risk of utilizing human antisera, as was done before.

The fundamental purpose of this project is the development of a prototype for the standardization of the production methodology and indutrial scaling up of monoclonal antibodies against various antigens relevant to human medicine, as well as offering to the market a commercial kit and defining the pre-scale up conditions defined as units needed to satisfy and/or conquer the national market. To that end cell culture and nuclear fusion techniques will be used, as well as antibody-producing hybridoma selection, commercial scale up (*in vivo* and *in vitro* cell culture, antibody purification and identification of classes and sub-classes of immunoglobulin), formulation of the prototype reagent and produce of an experimental batch for product testing.

At present we have several monoclonal antibodies-producing hybridoma cell lines against

A and B red blood cell antigens. These antibodies have been characterized with respect to their isotypes, specificity. The results indicate that these antibodies could be used for the formulation of a commercial kit for haemoclassification.

Additionally we are working on the production of these antibodies *in vitro* and in vivo systems in mice and on the formulation of a final product with the required specificity, sensitivity and stability.

DESIGN OF DNA PROBES FOR PHENYLKETONURIA DIAGNOSIS

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(*) Until year 1994

OBJECTIVES

- Metabolic disorders that for their high frequency or because being new mutations, not identified in other populations, might represent a comparative advantage in our country.
- To implement methods for the identification of phenylketonuria carriers and for the detection of new mutantions, starting with families where the disorder has been identified.
- To extend earlier studies of high risk populations, basically interns with mental retardation, showing symptoms of phenylketonuria.
- To make advances in the study of metabolic disorders in our country by correlating data on enzymatic activities with possible errors in the DNA.
- To establish the most adequate methodologies for future intrauterine early diagnosis of this and other innate metabolic disorders.

SUMMARY

Phenylketonuria is an innate disorder of phenylalanine metabolism, it is autosomic recessive and is caused by a deficiency in the enzyme phenylalanine hydrolase (PAH). The main symptoms are hyperphenylalaninemia, neurologic disorders, mental retardation, and dermatologic problems, if not diagnosed and treated timely. Taking into consideration the polymorphism of the PAH gene (more than 100 different mutations) and the correlation with the geographic and ethnic origin of some populations as well as the possible correlation of the mutation with the severity of the sickness, the study of the Colombian population is of great interest from the clinical and anthropological point of view.

In this project we studied ca. 3500 patients with mental retardation, whereby 4 families presenting phenylketonuria with clinical manifestations and phenylalanine levels differering from typical pathology were detected. Using the polymerase chain reaction (PCR) methodology we amplified exon 3 and introns 10 and 12 of the gene PAH, extracted from leukocytes of 7 patients, 5 heterocygotes and 120 controls, thereby obtaining amplified segments of 302 bp (exon 3), 350 bop (intron 10) and 375 bp (intron 12). These segments were hybridized to ASO probes in the following manner: exon 3 (haplotype 4) was hybridized to the ASO probe for the mutation R111X, predominant in the oriental population; intron 10 (haplotype 6) with ASO probe for mutation IVS 10, predominant in the Mediterranean population; and with ASO probe for mutation IVS 12 for intron 12, predominant in northern Europe. The results were negative for the population studied. This indicates that the mutations present in Colombian

patients differs from that most frequently found in phenylketonuric patients from Argentina, Chile, Mexico and Spain.

We have not obtained hybridisation signals in genomic DNA, RFLP analyses of hyperphenylalaninemic patients and their relatives using the restriction enzymes EcoRI, EcoRV, HindIII, MspI and PvuII utilizing a PAH cDNA clone.

The project permitted the implementation of the molecular biology laboratory of the Biochemistry Research Center, as well as the implementation of the following techniques: DNA extraction from blood samples and cell tissue culture, DNA electrophoresis in agarose and polyacrylamide gels, DNA digestion with restriction enzymes, Southern and dot blot analysis, radioactive labeling of DNA and plasmid purification, PCR, RFLP and SSCP analysis, silver staining of gels and hybridisation techniques, in which we started the study of the enzymes responsible for mucopolysaccharidosis types I and IV and Tay Sachs syndrome.

EXPRESSION MAPS, TRANSCRIPTION ASSAYS AND SUBTRACTIVE GENE LIBRARIES IN *Saccharomyces cerevisiae* UNDER STRESS CONDITIONS. A PROPOSAL FOR THE DEVELOPMENT OF A METHODOLOGY

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OBJECTIVES

- To define the conditions for the resolution of *Saccharomyces* chromosomal fragments utilizing pulsed field gel electrophoresis (strain AB972) and to generate a set of representative membranes of each chromosome for Southern blot analysis.
- To carry out transcritional studies in *Saccharomyces cerevisiae* grown in YPD at 33 and 39°C in order to produce labeled RNA under both growth conditions.
- To localize gene loci in *Saccharamomyces cerevisiae* that are expressed under both growth conditions by hybridizing the blots with RNA from the transcriptional studies.
- To compare the resulting band patterns (size, localization of the bands and intensity of the hybridisation signal) in order to be able to define which bands show differences.
- To construct subtracted cDNA libraries starting from *Saccharomyces cerevisiae* mRNA grown in YPD at 33 and 39°C and subtracted in both directions (with RNA in excess) in order to isolate characteristic messages, and if present, also those specific for cells grown at both temperatures.
- To hybridize chromosomal Southern blots of *Saccharomyces cerevisiae* with labeled fractions of subtracted libraries.

SUMMARY

The methodologies used at present to sequence a genome do not make completely clear how to interpret the sequence and physical structure in terms of cellular processes. The sequence of a gene can be determined, but it is not quite clear how to define its role. For this reason it becomes necessary to study genetic changes *in vivo* at the very moment they happen to give us a complete and precise map of cellular changes caused by cellular events.

In order to make these technologies independent from the existence of a probe or antibody they must possess the following two characteristics: the first is that they must allow a reproducible mapping of each chromosome of the organism at a higher resolution level than at present, and secondly they must be able to detect the genes expressed as the result of an induction event.

In this project we propose to localize and quantify the level of expression of *Saccharomyces cerevisiae* strain AB 972 using pulsed field gel electrophoresis and transcriptional studies (nuclear transcription run-off assays) and probes from subtracted cDNA libraries enriched for event-specific messages.

So far we have conducted 29 pulsed field electrophoresis experiments with the *S. cerevisiae* strains UN and UA. All experiments were performed using a commercial electrophoresis apparatus (Hula Gel, Hoefer). This machine, developed by De Southern, allows to physically re-orientate the gel with respect to the electric field, thereby interrupting the current during re-orientation, and therefore obviating the need to correct for the passage of electrons using the modifications as proposed by Chu in the CHEF (Contour-Clamped Homogeneous Electrophoresis Field) methodology.

PRODUCTION OF THE MAJOR ALLERGEN FROM THE MITE *Blomia tropicalis* USING RECOMBINANT DNA TECHNOLOGY

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OBJECTIVES

- To clone the gene of the major allergen from the mite *Blomia tropicalis*.
- To sequence the cloned gene and to infer the primary sequence of the protein (allergen) codified by this gene.
- To purify the recombinant allergen.

SUMMARY

The mites *Blomia tropicalis* (*Bt*) and *Dermatophagoides pteronyssinus* (*Dp*) are an important source of allergens capable of sensitizing a high proportion of the population. Among the allergic population genetic markers, such as immunoglobulin allotypes of the major histocompatibility complex (HLA), have been studied, and very interesting results concerning a possible genetic control of allergic phenomena have been obtained (especially the one associated with allergic asthma). Detailed immunochemical and molecular studies are required in order to determine the genetic control of the IgE response at the level of allergenic epitopes and antigenic presentation in vitro to confirm or enable the knowledge of the key role played by HLA in allergic processes.

We constructed a cDNA library of the mite *Blomia tropicalis* (*Bt*) in phage Lambda gt11 and expressed it in *E. coli* strain Y1090. Several recombinant allergens were subcloned for expression and composition analysis, as well as for DNA and aminoacid sequence analysis in different plasmid, such as pBlueScript, pCRII, pGMEX and pGEX, which are maintained in different transformed *E. coli* strains.

Five recombinant clones that codify native allergens have been isolated from this library (BtM, BtIIa, Bt2, Bt6b and A2a). It is expected that the library will contain other recombinant allergens, which will be detectable using other probes, be it antisera (IgE from allergic patients) or with probes designed based on the sequences already obtained.

Furthermore, we have purified two recombinants (BtM abd BtIIa) which correspond to the most important allergens involved in the induction of allergic asthma. The others have been partially characterized with respect to DNA sequence and percent binding to IgE.

STANDARDIZATION AND APPLICATION OF *IN SITU* HYBRIDIZATION FOR THE DIAGNOSIS OF GENETIC DISORDERS

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OBJECTIVES

- To standardize the *in situ* hybridisation technique.
- To identify and diagnose chromosomatic anomalies (minichromosomes and infrachromosomic anomalies)
- To localize regions with chromosomal fragility and structural alterations of oncogenes and rupture points involved in some neoplastic chromosomal rearrangements.

SUMMARY

The *in situ* hybridization (ISH) combines cytogenetic and molecular biology techniques, allowing the localization of physical chromosomal regions with highly repetitive DNA sequences, hypervariable regions, genes implicated in pathological disorders and pseudogenes. Similarly, it allows in detail study of genetic maps, evolutionary mechanisms, hereditary disorders and cancerogenic processes.

This project includes lymphocyte and tissue culture and/or the establishment of lymphoblastoid lines from individuals or families with chromosomal inframicroscopic anomalies for the obtention of chromosomes. These chromosomic preparations will be employed for in situ hybridization with enzymatically or radioactively labeled probes, depending on the type of probe.

During the project we were able to standardize the *in situ* hybridization technique on healthy individuals utilizing centromeric and total chromosomal probes labeled with digoxigenin. After that we analysed patients with chromosomal anomalies (partial and universal trisomy, translocations, isochromosomes and isodicentric chromosomes, male 46 XX phenotypes and minichromosomes) to confirm and/or determine these findings obtained using conventional cytogenetic techniques using ISH.

IV. INDUSTRIAL BIOTECHNOLOGY

ENHANCEMENT OF SOLVENT PRODUCTION BY *Clostridium acetobutylicum*

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OBJECTIVES

- Organization and handling of *Clostridium acetobutylicum* strain bank.
- Isolation of indigenous strains of C. *acetobutylicum* from Colombian soils.
- Isolation by transconjugation of mutant C. *acetobutylicum* resistant to butanol.
- Study of butanol-resistance mechanisms through function and composition studies of cell membrane in C. *acetobutylicum* DSM 1732 and spontaneous mutant individuals butanol-resistant.
- Isolation of hydrogen hiper-producing C. *acetobutylicum* mutants.

SUMMARY

The organization and handling of *acetobutylicum* strain bank was developed by establishing procedures for growth, preservation, maintenance and reactivation for different strains obtained by isolation, or the ones developed by spontaneous mutation or by transposition. This work also includes a revision viability program in order to control the quality of preserved material.

The indigenous strains of C. *acetobutylicum* were isolated from 155 Colombian soil samples. 178 isolations were made, and evaluated for acetone production (100 showed a strong reaction and 78 a weak reaction); for butanol production (100 produced more than 2 g/L). A higher total solvent production was found in soils where tubers are grown (9.5 g/L), followed by soils planted with coffee (7.7 g/L), and grass (7.5 g/L). In terms of soil pH, isolations from acid soils are 63.64%, with a total solvents media global production of 6.0 g/L; while neutral pH are 36.36%, with a 6.6 g/l. Only 8.5% have a higher production of total solvents than the reference strain, *acetobutylicum* ATCC 824 (14 g/l total solvents). Two of the isolations (66A and 125C) exceed the butanol production of ATCC 824 strain (8.4 g/L). Isolations 18A and 63D exceed the acetone production of ATCC 824 strain (4.2 g/L). Isolations that show evidence of activation (turbidity and gas production) before 24 hours from incubation have a higher probability of becoming solvent producers, not as the isolations that activate after 48 hours of incubation.

In obtention of mutants by transposition, 451 eritromycin- and butanol- resistant (25 g/L and 1.25% respectively) were recovered, from 29 transconjugation events. 110 out of these, were activated by thermal shock (70 C/10 min), and 95 of them are sporulating mutants. 58 out of the 95 isolations are tetracyclin-resistant (10g/mL). 9 out of these 58 resist 2% p/v butanol, 8 resist 1.8% p/v, 29 resist 1.25% and 12 resist 1.0% butanol. Among the butanol-resistant mutants there are solvents hyper-producers and hipo-producers. 63.8% of the mutants exceed ethanol production,

5% acetoneproduction and 10.3% butanol production, compared to *acetobutylicum* ATCC 824. For example, mutants 21K1 and 22A9 produce 19.9 and 19.2 g/L total solvents respectively.

The study of membrane lipids and fat acids was undertaken in butanol-resistant C. *acetobutylicum* DSM 1732, ATCC 824 and three spontaneous DSM 1732 mutants. The results showed a third phospholipid, different from the two reported by literature, and it was not possible to determine its structure in DSM 1732 strains and their mutants. The total phospholipids contents is higher in the mutant strains than in the other two. The presence of fat acids C12, C14, C16, C18, C18:2 and C18:1 was detected in all strains. C16:1 was detected in 04M, C22 in DSM 1732 and 10 AN; and C26 in 10 AN. The contents of saturated and non-saturated fat acids decreases after 48 hours in DSM 1732 and O4M strains, while in 10 AN strain it increases. The proportion saturated fat acids vs. non-saturated fat acids increases within 48 hours and decreases again after 72 hours.

In terms of pH in C. *acetobutylicum* cultures (DSM 1732 and three mutants), the results showed that pH is higher for mutant strains than the wild strain. Its highest peak appears at 24 hours, when the shift to solventogenic phase happens. This value is higher for the hyper-producer mutant strain (10 AN), and it continues to be the highest after 48 hours.

The results for butanol-resistance mechanisms showed that the membrane initial composition enables the microorganism to stand high butanol concentrations and, additionally, the strain must be able to respond to solvent attack, represented by a membrane composition change. This change implies the synthesis of new lipids, an increase of phospholipid contents, and the possibility of increasing saturated fat acids contents at the expense of non-saturated fat acids. This enables the microorganism for supporting a high gradient of transmembrane pH, and thus accumulate high solvent concentrations.

EXTRACTION AND CHARACTERIZATION OF PECTINS FROM TROPICAL FRUITS (*)

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(*) Project co-Supported by COLCIENCIAS and by the Multinational Biotechnology and Food Technology Program (PMBTA, OEA).

OBJECTIVES

- To isolate the pectins and its sub-products from the Castilla berry (*Rubus glaucus*) and from Uchuva (*Physalis peruviana*) to develop an industrial production process.
- To characterize the pectins and its sub-products from the Castilla berry (*Rubus glaucus*) and from *Physalis peruviana*.
- To determine the effects of pH, temperature, and heating time on the extraction process and quality of pectins extracted from the Castilla *berry (Rubus glaucus)* and from Uchuva (*Physalis peruvian*).
- To design laboratory scale processes utilizing residues stemming from the processing or handling of the fruits (fruits discarded during the selection process because of mechanical damage and other quality factors such as size, variety, ripeness, etc or production processes such as peels, pulp extraction residues, and pulp refinement).

SUMMARY

Colombia invests a considerable amount of money to import raw materials, among them pectins, while it possesses a great variety of natural perishable products that contain pectins, especially fruits. These are wasted in great quantities because of the lack of utilization alternatives. Besides, produced increasing the volumes of contaminating organic matter that must be disposed, thereby increasing the costs of production and the negative effects on the ecosystem.

Pectin production utilizing industrial fruit residues is a possible solution to this problem and is directed toward the reduction of losses through rational use of processing residues and its commercialization.

The purpose of this research is to characterize the pectins and their subproducts from the Castilla berry *(Rubus glaucus)* and the uchuva fruit *(Physalis peruviana)* with the purpose of design industrial production processes. Additionally, the effects of the pH, temperature and time of the extraction process on the quality of the pectins are discussed.

For the Castilla berry two pH values were used: 2.2; 3.6 and hydrolisis times of 45 and 75 minutes with fruit extracting media weights relations of 1 :1 and 1 :5 were made. Two extractions were made in each case. The pH values were obtained using hydrochloric acid and soda for pH 3.6. Eleven batches were run and the yields 0.390% w/w to 1.366% w/w were obtained.

To reduce the color produced by the antocianins of the berry several treatments were made with diatomaceus earth, redispersion in water and reprecipitation, filtration through activated charcoal and Dowex 50 wx4 cationic resin.

The samples were characterized using the WHO and FAO standards procedures including a commercial pectin: Unipectine RS 150.

The pectin samples (11) from Castilla berry showed that:

1. The highest yield was got with pH 3.6; 45 min. hydrolisis time and 1 :3 w/w fruit; extracting media relation.
2. A second extracting procedure is not recomended by economical and technological reasons.
3. The sodium hexametaphosphate did not increase the pectin yield.
4. The Dowex resin did not removed completely the color compounds.
5. The pH 3.6 give the highest yield, equivalent weight and the lowest free acidilyt values.
6. The best conditions for extracting high methoxyl pectins (HMP) were: pH 3.6; 45 min hydrolisis and 1 :1 w/w fruit extracting media. At pH 2.2 low methoxyl pectins were obtained.

The pectin from the uchuva fruit *(Physalis peruviana)* showed that:

1. The best conditions for extraction were: pH 3.2; 75 min at 90°C hydrolisis and relation 1 :1 w/v fruit extracting medium.
2. There is a clear relationship between the extraction conditions and the quality of the pectin obtained.
3. The dictomaccus carth, sodium hexametaphosphate and Supercel used in the extracting procedures did not improved the pectin quality.
4. The uchuva pectins obtained (10 batches) proved to be of low methoxyl content (<7%), rapid setting so that can be used for low calorie foods. Their viscosity properties did not change with the extraction conditions.

EXTRACTION AND CHARACTERIZATION OF *Physalis peruviana* PECTIN (*)

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OBJECTIVES

- Characterization of pectins and process sub-products of *Physalis peruviana*, for designing alternative processes for their industrial production.
- Characterization of pectins and process sub-products of *Physalis peruviana*.
- Determination of the influence of parameters such as pH, temperature, heating times, on *Physalis peruviana* extraction process and quality.
- Design of processes, at both laboratory and pilot plant levels, using process and handling residues as raw material (residues as discarded fruit in selection processes due to mechanical damage or quality factors as size, variety, ripeness, etc. or as skin, pulverizing and pulp residues).

SUMMARY

The food industry, as well as the pharmaceutical industry, needs increasing quantities of pectin for their industrial processes. But the quality and behavior of the substance are not widely known, leading to a quality loss and waste of the product.

This project seeks to extract and characterize *Physalis peruviana* pectin. Different extraction systems were tested, modifying pH, hydrolysis time and the weight proportion fruit: extraction medium. Color and produce were tested with sodium hexametaphosphate, supercel and diatomeaceae soil. A quality evaluation showed the ash contents, equivalent weight, free acidity, methoxile percentage, sterification degree, anhydrouronic acid contents, calcium, magnesium, iron, viscosity and jelling degree.

The optimal conditions were: pH 3.2, hydrolysis time of 75 minutes, and 1:1 fruit:extraction medium proportion. The obtained pectin showed the following characteristics: low methoxile, many sterified groups, quick decantation, and can be used for low-sugar jelly production, due to its gelling with 35% of soluble solids.

EXTRACTION AND CHARACTERIZATION OF *Rubus glaucus* PECTIN (*)

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OBJECTIVES

- Characterization of pectins and process sub-products of *Rubus glaucus*, for designing alternative processes for their industrial production.
- Characterization of pectins and process sub-products of *Rubus glaucus*.
- Determination of the influence of parameters such as pH, temperature, heating times, on *Rubus glaucus* extraction process and quality.
- Design of processes, at both laboratory and pilot plant levels, using process and handling residues as raw material (residues as discarded fruit in selection processes due to mechanical damage or quality factors as size, variety, ripeness, etc. or as skin, pulverizing and pulp residues).

SUMMARY

In order to benefit from the wide variety of the country's perishable products, and taking into account that *Rubus glaucus* presents good agricultural features for developing agroindustrial products, the objective was to extract and characterize *Rubus glaucus* pectin, standardizing the process conditions related to pH, hydrolysis time, pulp-water proportion and its influence in quantity and quality of the obtained pectin.

The most efficient extracting method should produce a high or low methoxile product, using as selection parameter the hydrolysis pH: pH 2.2 for low methoxile pectin, and pH 3.6 for high methoxile pectin. The extraction conditions for a high methoxile pectin, with quick decantation time and high gelling power, according to WHO's and FAO's international standards were: pH 3.6, hydrolysis time of 45 minutes, and water:pulp proportion 1:1 weight/weight.

OBTENTION AND CHARACTERIZATION OF PECTIN FROM INDUSTRIAL RESIDUES OF MANGO (PEEL) (*)

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OBJECTIVES

- To determine the optimal extraction conditions of pectin from mango peel and charaterize their quality.

SUMMARY

The objective of this project was to establish the optimal pH and hydrolysis time for the obtention of pectins from mango (Mangifera indica) peel and determine the effect of sodium hexametaphosphate as a hydrolysis adjuvant during the extraction process.

The raw material for this investigation was obtained from industrial mango processing residues. Pectin was extracted from the exocarp using an acidic extraction protocol at pH 3.2, 3.4 and 3.6, and hydrolysis times of 45, 60 and 75 minutes, to obtain the highest yield and quality of pectin. Parameters analysed were: humidity, ashes, ash alkalinity, equivalent weight free acid, esterification degree, rheologic characteristics, hyaluronic acid content, calcium, magnesium and iron contents.

Our results showed that the best extraction conditions were, pH 3.2 and a hydrolysis time of 75 minutes, and that the addition of sodium hexametaphosphate increases the extraction yield 23%, but it also doubles the requirement for hydrochloric acid for the hydrolysis.

Additionally we found that it is possible to obtain high quality pectins from mango peels stemming from industrial processing (residues represent 50% of biomass), as compared to a commercially used pectin that was taken as the standard.

Most extracted pectins had low levels of methoxy groups, thereby rendering them useful for the production of gels with low soluble solids content (sugar) and therefore of low calory alimentary products (marmelades and jellies).

The best pectin extract was obtained hydrolysing at pH 3.2 after 75 minute of heating, including sodium hexametaphosphate. Under these conditions we obtained the highest yield of anhydrouronic acid (AUA) and the highest overall yield (23-24%), although the amount of hydrochloric acid required for the hydrolysis was doubled.

The residues produced during the extraction of pectins could be further used as raw material in the production of animal feedstuff.

PECTINASE PRODUCTION IN IMMERSED FERMENTATION UTILIZING CITRIC RESIDUES AS SUBSTRATE (*)

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(*) Project co-Supported by the Multinational Program for Biotechnology and Food Technology (PMBTA, OEA).

OBJECTIVES

- To develop a production process for fungal pectinase using immersed fermentation, utilizing citric residues as a substrate.
- To determine the composition of the citric residues to be utilized as substrate.
- To design the culture media for the production of pectic enzymes in immersed fermentation.
- To evaluate the potential inductory effect of the substrate at laboratory scale.
- To select the strain to be used based on the characterization of pectinases and to evaluate its production potential.
- To establish the most adequate conditions for the purification of the enzyme obtained from immersed fermentation.

SUMMARY

The objectives of the present project were to develop a production process for fungal pectinase using immersed fermentation, utilizing citric residues as a substrate, to design the culture media for the production of pectic enzymes in immersed fermentation, to evaluate the potential inductory effect of the substrate and to establish the most adequate conditions for the purification of the enzyme obtained from immersed fermentation. To that end we studied the kinetic parameters of strain 7-121 of the fungus *Trichoderma auriviridae* and the mutant strain CH4 of the fungus *Aspergillus niger*. The substrate utilized was obtained from the solid residues stemming from the processing of oranges var. Valencia. The evaluated parameters were, cell concentration, ammonium sulfate concentration, extracellular protein, pectin esterase (PE) and exo-poygalacturonase (EXO-PG) activity.

The growth and production kinetic studies showed in all cases that protein production was associated to cell growth and an increase in EXO-PG production that reached its peak during stationary phase growth that diminished afterward with the concomitant increase of PE activity. It became evident that growth was limited by carbon supply from the citric residues and the pectin, yielding ca. 3 g cell mass/gram of ammonium sulfate.

The highest pectinase yield (PE and EXO-PG) was obtained using automatic pH control. Using this methodology we also obtained the best enzymatic activity for PE and EXO-PG.

V. LIVESTOCK BIOTECHNOLOGY

DEVELOPMENT OF A NEW DIAGNOSTIC METHOD FOR THE DETECTION OF BOVINE TUBERCULOSIS UTILIZING THE POLYMERASE CHAIN REACTION (PCR)

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* Hasta 1996

OBJECTIVES

- To develop a new diagnostic method utilizing the polymerase chain reaction (PCR) to evaluate the presence of bovine tuberculosis in human and animal populations and to detect *Mycobacterium bovis* in biopsies and secretions of humans who are suspected to suffer from a zoonotic tuberculosis.
- To identify a species-specific nucleotide sequence in *Mycobacterium bovis* not present in other mycobacteria or other related bacteria.
- To isolate and to characterize sequences species-specific sequences utilizing conventional molecular biology tools.
- To sequence the whole selected genomic DNA fragment using the enzymatic dideoxynucleotide sequencing methodology.
- To design and chemically synthesize the oligonucleotides that will serve as PCR primers.
- To standardize the PCR conditions using *M. bovis* DNA and the primers synthesized above.
- To determine the specificity of the PCR reaction using DNA isolated from other mycobacteria species and other microorganisms implicated in the differential diagnostics of bovine tuberculosis.
- To determine the detection limit using highly purified *M. bovis* DNA and milk samples previously contaminated with the bacterium

SUMMARY

Bovine tuberculosis is currently an important zoonosis around the world and the infection of humans with *Mycobacterium bovis* cannot be ignored. Epidemiologic information is scarce, but it is thought that ca. 6-30% of all human tuberculosis in the US were caused by *M. bovis*, before pasteurization of milk, as reported by Karlson and Carr in 1970. It is also the cause of 6.3% of all confirmed bacteriologically confirmed cases in Ireland. Furthermore, in a study conducted in New Zealand, an increase in bovine tuberculosis was found between the years 1983 (3.7%) and 1989 (14.6%).

Inspite of the fact that in some regions of Latin America diagnosis is still conducted in the traditional way, e.g., direct plating of clinical samples, it is estimated that ca. 7000 new cases of bovine origin tuberculosis were diagnosed in humans in 1991, according to data from the Panamerican Health Organization.

To prevent humans from contracting the disease it is essential to control and erradicate *M. bovis* in cattle. Nevertheless, erradication campaigns have not been satisfactory due to the low sensitivityand specificity of diagnostic skin assays usually employed for the detection of infected animals. Hence, not having a precise diagnostic method for the detection of the causal agent in cattle leads not only to an increase of infection on humans, but also to economic losses in the livestock industry.

Species-specific identification of mycobacteria for conclusive diagnosis has always been difficult. Different serologic approaches have been tried, but have failed mainly due to their low sensitivity and specificity and the high level of cross reactivity with other mycobacterial antigens. Recently, molecular biology tools have proved to be highly valuable in the diagnosis of mycobacteria, including the distinction of species, whereby PCR technology plays a pivotal role herein. Most tests amplify regions of the so-called tuberculosis complex, and therefore they cannot distinguish between infections caused by *M. tuberculosis* from those caused by *M. bovis*.

Methods for the specific identification of *M. bovis* usually require a previous amplification of a fragment common to all mycobacteria and sub-sequent specific detection using a radiactive labeled probe or restriction enzyme analysis. As a consequence of the high antigenic cross-reactivity and the close genomic relationship between mycobacteria, the identification of a species-specific sequence becomes a difficult task.

Our group has previously reported the use of PCR for human tuberculosis diagnosis using a species-specific *M. tuberculosis* sequence. In this study we describe the use of the RAPD technique for the identification of a species-specific *M. bovis* genomic fragment and the development of a one-step PCR assay that could yield a powerful tool for epidemiologic studies that would lead to an efficient control of bovine tuberculosis.

RAPD (Random Amplified Polymorphic DNA) was employed for the identification of a species-specific *Mycobacterium bovis* fragment. A 500 bp fragment was amplified from 15 different *M. bovis* strains, including *M. bovis* BCG strain Pasteur, which was absent in 26 different mycobacteria strains and 20 clinical isolates of Mycobacterium tuberculosis. This fragment was the used as a probe in a Southern analysis showing different signals common to M. tuberculosis and *M. bovis*. A 2.9 Kbp fragment was identified that was specific to *M. bovis* but was absent in *M. tuberculosis* and *M. avium*. Using partial sequence information from the 500 bp fragment a piar of primers was designed for PCR. Using pure samples of DNA extracted from different mycobacteria, only *M. bovis* and *M. bovis* BCG produced an amplified fragment. This technique is capable of detecting down to 10 fg pure *M. bovis* DNA, which is equivalent to finding 3 bacilli in the sample. .This assay will be useful in detecting non-cultivated bacilli stemming from clinical samples, like milk.

Identification of a species-specific fragment. In order to identify a species-specific fragment a RAPD analysis was conducted using DNA from *M. bovis* and *M. tuberculosis* (the nearest related species) as templates. RAPD primers were used individually or in combinations. Many fragments became visible on agarose gels, but most of them were common to both species. Primer JB2 produced a band of 500 bp which was present in three different isolates of *M. bovis* but absent from *M. tuberculosis* strain H37Rv. To determine whether the fragment was specific to *M. bovis* we used the JB2 primer to amplify 22 different mycobacterial strains and three different *M. bovis* isolates. In this experiment many bands amplified in the different strains made a clear identification of the previously identified 500 bp fragment very difficult.

The amplified fragment were transferred to nylon membranes and hybridized to the radiolabeled *M. bovis* 500 bp fragment. A positive signal was obtained only in the *M. bovis* lanes but not with the other mycobacteria. The fragment was then used to conduct a Southern blot analysis on EcoRI digested genomic DNAs from *M. bovis*, *M. tuberculosis* and *M. avium*.

The autoradiography revealed homologous regions between *M. bovis* and *M. tuberculosis*. Nevertheless, the 500 bp probe also identified a 2.9 Kbp fragment only present in *M. bovis* with no homolog in *M. tuberculosis* and *M. avium*.

M. bovis-specific PCR. The *M. bovis*-specific fragment was cloned and the ends were sequenced. Based on this sequence information, primers JB21 and JB22 were designed, synthesized and purified.

Using these primers we optimized the conditions for the PCR reaction, using as a template DNA from *M. bovis* and *M. tuberculosis* strain H37Rv. After 30 amplification cycles a 495 bp fragment was observed exclusively for *M. bovis*. No amplification products were observed using any of the other genomic DNAs.

Specificity and sensitivity of the PCR reaction. The specificity of the reaction was evaluated using DNA from 14 reference mycobacterial species. The 495 bp amplification product was found exclusively in the case of *M. bovis*. To demonstrate the usefulness of this assay in distinguishing *M. tuberculosis* from *M. bovis* we tried 20 clinical M. tuberculosis isolates and 11 *M. bovis* isolates using primers JB21 and JB22. A positive signal was obtained only for the *M. bovis* isolated, thereby confirming the previous results obtained the reference strains.

To demonstrate that the PCR assay can be used directly on clinical samples, 1 mL milk was contaminated with *M. bovis* as well as with bacteria commonly found in milk. Again, the amplification product was only detected in samples contaminated with *M. bovis*, demonstrating that the assay is not only specific, but that milk components are not inhibitory to the assay.

The sensitivity of the technique was tested using decreaing amounts of DNA. Down to 10 fg of *M. bovis* DNA were detected as seen by visualizing the amplified bands on agarose gels.

In spite of the fact that there exists a high degree of homology between the various mycobacteria species, especially among those belonging to the tuberculosis complex (which *include M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum* and *M. microti*) we were able to develop a new, species-specific method that was able to detect exclusively *M. bovis*. In this study we combined the use of two common molecular biology techniques to identify a species-specific genomic DNA fragment from the bovine bacillus: using the RAPD technique we identified a unique *M. bovis* fragment, which was then used to generate a pair of specific primers for PCR analysis. RAPD has been previously used for the typification of strains from various microorganisms, including *M. tuberculosis*. The approach described here allowed us to distinguish *M. bovis* from other mycobaterial species, and more important, from other strains belonging to the tuberculosis complex, which share more than 90% homology at the genomic level.

Southern blot analysis using the 500 bp fragment detected 4 fragments coomon to *M. bovis and M. tuberculosis*, suggesting the presence of partially homologous regions, which confirms the close relationship between these two species. A strong hybridization

signal with a 2.9 Kbp fragment was only detected in the case of *M. bovis*, suggesting a polymorphic region that could be used in restriction pattern analysis recognition. We are presently analysing whether this fragment is part of a previously reported repetitive sequence or an intergenic sequence between these repetitive sequences.

The PCR assay utilizing primers JB21 and JB22 clearly amplifies a fragment unique to *M. bovis*. This method represents an enormous advantage over previously reported techniques which involve an amplification step followed recognition with differential probes or using restriction enzymes. Due to the aggregation phenomenon characteristic of mycobacteria we were not able to determine the number of mycobacteria in a contaminated milk sample. Nevertheless, we were able to detect down to 10 fg of DNA, which is equivalent to 3 bacilli according to previous reports.

The new diagnostic method based on the amplification of a genomic *M. bovis* DNA fragment using PCR, will allow direct identification of the bacillus in biological samples, such as milk, making it a good assay for rapid diagnosis, sensitive and specific for *M. bovis*. Early detection of *M. bovis* by PCR can be very useful as en epidemiologic tool for field analysis of cattle or in programs in human clinical cases to be able to determine the real magnitude of bovine tuberculosis.

Section two

APPROVED PROJECTS INITIATING EXECUTION

I. AGRICULTURAL BIOTECHNOLOGY

APPLICATION OF SOME BIOCHEMICAL AND MOLECULAR RESISTANCE MARKERS IN THE ANALYSIS OF THREE ESPECIES OF *Solanum Lam* GENUS

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OBJECTIVES

- Analysis of the ability to develop systemic resistance in three species of the *Solanum Lam* genus (*S. quitoense*, *S. candidum* and *S. pseudolulo*) to *Fusarium oxysporum* and RNA Potyvirus, using as parameters the following biochemical biomarkers: production of B 1-3 acid glucanases; production of peroxidases and production of calose (B 1-3 glucane).
- Determination of the different resistance or susceptibility level of these three species, based on the analysis of the three biochemical markers.
- Identification and characterization of pathogen RNA potyvirus of the studied species.

SUMMARY

The *Solanum* fruit, lulo, is a native species from the Andean region. It has a good potential as fresh and processed fruit, but its production is restricted by its susceptibility to pathogens and its rough domestication. Some species related to lulo are not commercial, but they have renowned characteristics for resistance to pathogens. Thus, they can be used for improvement of lulo species. This project aims to generate the ability to develop systemic resistance in three species of the genus *Solanum Lam*, using biochemical markers as analysis parameters.

The results obtained show that the B 1-3 glucanases and the calose can be resistance markers in the mentioned genus, as they present a significant and constant growth in their levels as an effect of elicitation, showing an adequate reproducibility in the assays performed at different times. The resistant species is *Solanum marginatum*, which shows higher basal levels of glucanase and calose compounds.

The electrophoretic analysis of double band RNAs patterns (dsRNA) and the RT-PCR demonstrated under low titles the presence of viral entities of the Potyvirus type. But there is no direct relation between the virus presence and the detection of symptoms. So it may be concluded that the detected virus does not correspond to the causal agent of the pathology "leaf yellowing" or "fanned leaf".

CONTRIBUTION TO THE DEVELOPMENT OF GENETIC TRANSFORMATION SYSTEMS FOR THE PRODUCTION OF RESISTANT PLANTS AGAINST THE COFFEE BERRY BORER *Hypothenemus hampei*

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OBJECTIVES

- To evaluate conditions for stable transformation and plant regeneration from coffee protoplasts
- To identify DNA clones for seed proteins by the screening of a cDNA library of coffee seeds of the Colombia variety.

SUMMARY

In 1988 the coffee berry borer (*Hypothenemus hampei*) appeared for the first time in Colombia. This insect only feeds and reproduces in the coffee bean, and causes devastating damage to the coffee seed. It can lead to crop losses of up 60%. Unlike other phytosanitary problems, in the case of the borer, there are no known sources of genetic resistance. Therefore, the mainstay for the control of the coffee berry borer is the use of some cultural practices and the use of chemical insecticides that can cause problems to the environment. For this reason, since the borer made is appearance in 1988 in Colombia, the National Federation of Coffee Growers, through CENICAFE, started an integrated pest management program (IPMP) to decrease the economical impact of the pest, and to minimize the possible impact of insecticide use on the coffee zones. This program includes, as a mid-term goal, the development of improved methods of cultural control and the development of biological control methods. However, the long-term goal is to produce insect-resistant plants through genetic transformation techniques, as a complement to the other control methods.

In order to accomplish this goal, since 1991 the Federation has been supporting several research projects to develop transformation protocols, and also to evaluate compounds potentially useful in the genetic control of the borer. The final objective of this program is to incorporate in the Colombia variety -which is highly productive and resistant to the coffee leaf rust- resistant genes against the coffee berry borer. The aim of the present study is to accomplish the regeneration of transgenic plants from protoplasts. it is also proposed to in initiate a project on the identification , cloning and characterization of coffee seed genes that subsequently could allow us to obtain specific promoters for the expression of resistant genes in the coffee seed.

CLONING OF THE 94 KDA TOXIN GENE OF *Bacillus thuringiensis* subsp. medellín IN A CYANOBACTERIUM FROM MOSQUITO BREEDING PONDS OF COLOMBIA.

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OBJECTIVES

- To clone and express the 98 kDa toxin gene from *Bacillus thuringiensis* ssp *medellin* in a native cyanobacterium species.
- To conduct a survey of cyanobacterial species in the main mosquito breeding areas where malaria is endemic in Colombia.
- To carry out ingestion and digestion studies with the most commmon cyanobacteria on *Aedes aegypti* and *Anopheles albimanus* larvae.
- To clone the gene of the 98 kDa protein from *Bacillus thuringiensis* ssp *medellin* in a selected cyanobacterium.
- To analyse the expression of the protein in the recombinant cyanobacterium.
- To determine the toxicity of the recombinant cyanobacterium on larvae of *A. aegypti* and *A. albimanus*.

SUMMARY

An inventoryu of the cyanobacteria from the mosquito larvae breeding ponds in malaria endemic areas of Colombia was performed. It was found that the most predominant cyanobacteria species were belong to the genera Synechocystis sp, Synechococcus sp., Mycrocystis sp., Gloeobacter sp and filamentous as Phormidium sp., belonging to the LPP group (Lyngbia, Plectonema y Phormidium), Oscillatoria sp., Spirulina sp., Anabaena sp. y Nodularia sp. The most widely distributed are Synechocystis sp., Synechococcus sp., Group LPP y Oscillatoria sp. found in five of the eight evaluated areas.

Cyanobacterial species maintanined in the culture collection are identified as Synechocystis sp. Synechococcus sp. and filamentous of the genus Oscillatoria and LPP group. Strains of Synechocystis sp. were among the most frequently found from most of the ponds, followed in frequency by strain of the LPP group and Oscillatoria sp.

We have observed in preliminary bioassays that the development of first *instar A. albimanus* larvae by feeding on Synechocystis cells with a daily addition of 3x108 cells per ml. mosquito larvae could pass to the second.

A. albimanus larvae biossays conducted with daily addition of the aquatic gram negative bacteria as, *Asticaccaulis excentricus*, *Caulobacter crescentus* and Ancylobacter aquaticus at concentrations of 1.5x107, 1.5x108 y 1.5x109 cells per ml indicated that the mosquito larvae can grow until third instar in the treatment containing 1.5x109 cells/ml of the bacterium *A. excentricus*.

Due to the fact that the literature has indicated that there are expression problems of gram positive bacterial genes in cyanobacteria, and based on the results of larval development with aquatic gram negative bacteria, the objective of the project is to introduce the gene Cry11Bb1 in *A. excentricus, A. aquaticus and C. crescentus* by using the vector pEA1. In order to achieve this objective a change of orientation in the construct pMAR3 has been performed. To complete the subcloning procedure we are actually working in three strategies to perform the ligation of the Cry11 Bb1 gene by blunt ends in one side of the vector pEA1 and by cohesive ends in the other. The expression and toxicity of the recombinant *A. excentricus, A. aquaticus* y *C. crescentus* with the plasmid pEA1 was evaluated. Mortality of third instar *Culex quinquefasciatus* larvae was observed to be over 80% 48 hr post treatment with 10-3 dilution of the bacterial cultures and close to 60% at the dilution 10-4, which demostrates the possibility to use these bacteria for mosquito control.

CHARACTERIZATION OF *Beauveria bassiana* AND *Metarhizium anisopliae* ISOLATES FROM CENICAFE COLLECTION

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OBJECTIVES

- Performing of a morphological, biochemical and molecular characterization of the fungi *Beauveria bassiana* and *Metarhizium anisopliae* in order to obtain improved strains to be used in an integrated pest management program for the control of coffee berry borer.
- Determination of the pathogenicity and viability of multisporic isolates obtained from different insect orders and reactivated on the coffee berry borer.
- Morphologically and biochemically characterization of the selected isolates.
- Selection of the best isolates, in morphologically and biochemically terms those that have good thermic and UV light resistance for the obtention of monosporic strains.
- Obtaining of recombinants, generated through protoplast fusion, with desirable characteristics.
- Molecular characterization of different isolates using RAPD analysis.

SUMMARY

The efficiency of biological agents depends on the knowledge about them, their biology, the environment and the mechanism of interaction between those and the host. The characterization of multiespore isolates of the entomogenous *Beauveria bassiana* (Bb), were characterized evaluating the variables: pathogenicity against coffee berry borer-CBB (PCB), TL_{50}, spores production (S), diametral growth (DG), spore size (SS), germination rate (GR), UV resistance (UVR) and temperature resistance (TR), and (RAPD's), enzyme production (EP) and starting time of enzyme reaction (TER). For *Metarhizium anisopliae* (Ma), the evaluated variables were PCB, GR, EP, TER, UVR y TR.

In a preliminary analysis, the isolates were classified according to PCB, establishing 4 groups: Group 1, PCB <25%; Group 2, 26% & 50%; Group 3, 51% & 80%, and Group 4, >80%. For Bb, in the Group 1, were Bb9009, Bb9010, Bb9108, Bb9115, Bb9120 & Bb9307 (Colombia - *Hypothenemus hampei);* Bb9017 & Bb9020, from other countries. Group 4, were isolates from Coleoptera: Bb9002, Bb9007, Bb9012, Bb9021, Bb9102, Bb9116, Bb9202, Bb9203, Bb9207, Bb9208, Bb9212, Bb9213, Bb9216, Bb9218, Bb9301; from Lepidoptera: Bb9018, Bb9019, Bb9112, Bb9204, Bb9205, and from Homoptera: Bb9027. Seventeen of those were isolated in Colombia. For Ma, most of them. belonged to Group 4, nine from Colombia (Coleoptera). Ma9220, from Australia, (Coleoptera), belonged to Group 3.

The analysis of variance of the groups showed the effect of them in the variables PCB, TL_{50}, S, DG, GR, EP & TER for Bb and the variables PCB y GR for Ma. The Tukey test (5%) showed that the Bb & Ma groups were different in PCB. The RAPD's analysis gave a clear separation among isolates for locality. This characterization allows identification of the strains and establish relationship among them. according to their biology and locality, define specificity and potential for the CBB control and other insects of economical importance.

DEVELOPMENT OF A DIAGNOSE METHOD FOR *Fusarium Oxysporum* f.sp. *dianthi* USING THE POLIMERASE CHAIN REACTION TECHNIQUE

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OBJECTIVES

- Development of a diagnose system for *Fusarium oxysporum* f.sp. *dianthi* using the polymerase chain reaction technique.
- Validation of PCR system using field samples obtained from different commercial farms throughout the Sabana de Bogotá.

SUMMARY

Fusarium oxysporum f.sp. *dianthi* (Fod) is the etiologic agent responsible for wilt disease in carnations, a disease that causes large economical loss to the flower industry worldwide. The early diagnosis of the disease, especially in the material to be propagated, is essential for the good management of the commercial culture. The diagnose methods available today are not entirely satisfactory due to their complexity, the time consumed and the difficulty with which they discriminate between the formae special *dianthi* from other formae speciales.

In the first phase of this project, using the random amplified polymorphic DNA (RAPD) technique, we were able to distinguish between fod and other formae speciales of *F. oxysporum* isolated from different hosts. This study led to the identification of four groups (I-IV), with different genetic patterns in the taxonomic group (Fod). Molecular hybridization analysis using fragments obtained from be used in the development of a PCR based molecular diagnostic tool for *F. Oxysporum* f.sp. *dianthi*.

In the second phase these genomic fragments will be cloned and sequenced. This information will lead us to design primers for the standardization of a multiplex PCR assay that could be enough to identify greenhouse isolates belonging to the *F. oxysporum* f.sp. *dianthi*. The developed test will be evaluated using the strain collection available at CorpoGen and other isolates obtained from field studies in farms from the Savanna of Bogotá.

DEVELOPMENT OF A DIAGNOSTIC SYSTEM FOR *Fusarium oxysporum* f.sp. *dianthi* USING MOLECULAR BIOLOGY TECHNIQUES

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OBJECTIVES

- To develop a quick, specific and sensitive diagnostic method for the fungus *Fusarium oxysporum* f.sp. *dianthi* using the polymerase chain reaction (PCR) and Random Amplified Polymorphic DNA (RAPD) in order to provide growers with a diagnostic tool for disease management.
- To standardize the RAPD technique and design RAPD primers able to distinguish isolates belonging to different races of *Fusarium oxysporum* f.sp. *dianthi* from other species and non-pathogenic isolates of the fungus.
- To find a correlation between banding patterns of selected isolates produced by specific primers and their pathogenicity using susceptible and resistant carnation varieties for that purpose.
- To use the primers selected in the first two steps and use them to sequence part of the amplified fragments in order to design longer primers that will make the assay more specific.

SUMMARY

The phytopathogenic fungus *Fusarium oxysporum* f.sp. *dianthi* is the causal agent of carnation wilt, a disease that causes great economic losses to export growers and that requires the application of fungicides and other chemicals —noxious to the environment for its control. Erradication of *Fusarium* is difficult because of its resistance structures, the most effective control is achieved through the use of pathogen-free propagation material. Conventional detection methods to determine the differential virulence of the pathogen are not entirely reliable and take approximately 20 weeks to produce results.

In this project we want to develop a quick, specific, and sensitive diagnostic assay for the determination of physiological races and pathogenic forms of *Fusarium oxysporum* f.sp. *dianthi* using the polymerase chain reaction (PCR) and Random Amplified Polymorphic DNA (RAPD) analysis.

DEVELOPMENT OF DIAGNOSE METHODS FOR *Passiflora edulis* SIMS POTYVIRUS, IN VALLE DEL CAUCA DEPARTMENT

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OBJECTIVES

- Obtaining highly sensitive diagnose methods combining immunocapture and PCR amplification for detection of *potyvirus* in passion fruit, in Valle del Cauca department.
- Purification of viral particles after virus growth in passion fruit plants.
- Obtaining specific monoclonal antibodies against this virus.
- Marking of highly sensitive diagnose methods combining immunocapture and PCR amplification for detection of potyvirus in passion fruit, in Valle del Cauca department.
- Purification of viral particles after virus growth in passion fruit plants.
- Obtaining specific monoclonal antibodies against this virus.
- Marking of monoclonal antibodies with synthetic DNA through direct covalent union between DNA and the antibody molecules.
- Use of the DNA-antibody complex for detecting passion fruit potyvirus through ELISA sandwich technique and DNA amplification by PCR.
- Capturing of the viral particles by using the alternative method of monoclonal antibodies, DNA reverse transcriptase and cDNA amplification by PCR (PCR-INIA).

SUMMARY

ELISA immunoassays have been widely used for virus detection in plants (Clark and Adams, 1971), however, their sensitivity and specificity has not been optimal, particularly used with polyclonal antibodies. Recently, the sensitivity of detection systems based on antibodies has been improved by the combination of monoclonal antibodies and specific antigens marked with a DNA molecule and posterior amplification of the marked DNA by PCR, a technique known as Immuno-PCR. Today, the DNA is joined directly to the antibody, instead of using chimaera streptavidine-A protein as ligante, reducing the assay's complexity and allows the detection of various antigens. This research aims to extend the use of this technique to the detection of plant virus, using as a model the *potyvirus* that infects passion fruit (*Passiflora edulis* var. Sims), a plant of economic importance in the Valle del Cauca depatment, and of high demand in international and domestic markets.

The research will start from healthy passion fruit plants, infected with *potyvirus* supplied by ICA (Colombian Institute of Agriculture) in Palmira. The presence of the virus, its purity and integrity, are tested with electronic microscopy. Electrophoretic analyses of the virus coat protein were carried out by using polyacrylamide gels with sodium dodecil-sulfate (SDS), using molecular weight standards known as

carbonic anhidrase 29.000, G3P 36.000, and 45.000 daltons ovoalbumen, showing bands at 45.000 daltons.

The preparation was purified with cesium chloride (CICs) and Bradford, Harlow and Layne methods determined the protein concentration. The immunization process was undertaken with 4 to 8 months old endogamic BALB/c mice (Zola and Brooks, 1982). The activity of existent antibodies in the serum is determined by indirect ELISA by antigen fixation to the plate (PTA-ELISA), using a mouse anti-Ig G marked with alkaline phosphatase as conjugated.

DEVELOPMENT OF POTATO LINES WITH POSSIBLE RESISTANCE TO *Tecia solanivora* AND *Phthorimmaea operculella*

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OBJECTIVES

- Establishment and standardization of transformation technique for potato, at least in one of the following commercial varieties: Parda pastusa and Diacol capiro, by using a transformation system mediated by *Agrobacterium* and a marker gene B-glucoronidase (GUS). After that, one of the potato varieties will be transformed by introduction of a Cry1Ab modified gene that codifies the proteic fraction of protein 125 kDa of Bt subsp Kurstaki.
- Determination of expression of toxin in the selected potato varieties, and evaluation of resistance against *Tecia solanivora* and *Phthorimmaea operculella*.

SUMMARY

Potato is an important constituent of the Colombian diet. In the country there are around 165.000 ha planted with potato, with approximately 90.000 families depending on that crop. It is the food product with the higher per capita consumption: 65 kg. Antioquia department produces 10% of the total potato production of the country, some 230.177 tons/year.

The potato's moth, pest caused by laepidoptera insects Gelechiidae *Tecia solanivora* and *Phthorimmaea operculella* destroys, in Antioquia department only, an average of 15% of potato crop, with a cost of approximately 6 million dollars/year. *P. operculella* is susceptible to the pesticidal action of Bt subsp kurstaki and *T. solanivora* is susceptible to the same subspecies and to a recombinant strain of E. coli that contains a Cry1Aa gene. But these two options do not represent a good alternative for pest control, as larvae can penetrate into the tuber and there they find shelter from the pesticidal action of the product.

Through genetic engineering techniques, the project seeks to obtain potato plants with a possible resistance to potato's moth. Towards the achievement of this objective, the group has established an adequate medium for regenerating leaf explants from potato commercial varieties Parda pastusa and Diacol capiro. The standardization of transformation methodology for both varieties has been completed, using vectors with B-glucoronidase as marker. Additionally, transformation assays have been performed with Agrobacterium strain LB4404 pKC2301, containing Cry1 A(b) gene of Bacillus thuringiensis.

EVALUATION OF Trichoderma STRAINS FOR THE CONTROL OF THE LEAF CUTTING *Atta cephalotes.*

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OBJECTIVES

- To evaluate *Trichoderma* species as potential antagonists to the fungus cultivated by *A. cephalotes*.
- To select a strain of *Trichoderma* with high growth rate and high inhibitory potential against the fungus cultivated by *A. cephalotes*.
- To evaluate several selected strains of *Trichoderma* against the fungus *A. cephalotes* using *in vitro* models.
- To evaluate several selected strains of *Trichoderma* using laboratory assays with established *A. cephalotes* colonies.
- To evaluate the best selected strains of *Trichoderma* using field bioassays.

SUMMARY

Leaf cutting ants (Hymenoptera: Formicidae) are considered as significant pest for economically important crops such as ajonjoli, palma africana, cocotero, yuca, caña de azucar, mango, cipres, naranjo, limonero, manzano). The control of this pest is achieved mainly thorught the use of chemical insecticides, however the complex behaviour and the number of galleries formed made this task difficult to be accomplished.

We investigated interaction of 19 isolates of *Trichoderma* sp. and 4 isolates of *Gliocladium sp.* against *Attamyces sp.*, symbiont fungus of *Atta cephalotes* cutting ants. The majority (82.6 %) inhibited *Attamyces sp.* mycelial growth, which was probably due to colonization ability and competition fór subtratum, known mechanisms of some species of this genus. *T. lignorum* (T-26) was the strongest inhibitor (51.23 %) following Tuckey ($P < 0.05$), with colonization achieving 50-60 %, possibly because inhibitory substances were present, such as enzimes, metabolites, antibiotics, peptides, volatile and non-volatile substances.

Strains of *Trichoderma sp.* and *Gliocladium sp.* interacting with *Attamyces sp.*, brought about cell wall turgescence loss, vacuolation and granulation of cytoplasmic material of the latter's hyphae, and eventually the disintegration of the attacked fungus' cell wall after 96 hours.

Scanning electronic microscopy showed that in interactions of T. lignorum (T-26) and Gliocladium (G-55) with *Attamyces sp.*, the first's (T-26) hyphae grew in association with the latter's, in a massive but loose winding. Nevertheless, in some of the vision fields studied, *Attamyces sp.* hyphae either appeared disintegrated, or their cell walls had lysed, suggesting action from some toxic substances or lytic enzimes. Furthermore, on *Attamyces sp.* hyphae, we observed a structure similar to a chlamydospore, that was not seen in *Gliocladium* (G-55) interactions, nor has been described in litterature. *Gliocladium* (G-55) microphotography showed protrusions that possibly facilitate contact between its hyphae and those of *Atta cephalotes* symbiont fungus.

EXPRESSION OF THE *Bacillus thuringiensis* subsp. medellín GENES IN TOXIC AND NON TOXIC *Bacillus thuringiensis* AND *Bacillus sphaericus*

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OBJECTIVES

- To construct transgenic entomopathogenic bacteria with improved characteristics.
- To clone genes from the *Bacillus thuringiensis* subsp. medellin in non-crystal forming and toxic *Bacillus thuringiensis* strains, and in toxic and non-toxic *Bacillus sphaericus* strains.
- To analyse the expression of the proteins in several constructs using western blot.
- To quantify the toxic activity of the recombinant bacteria.

SUMMARY

Once concluded the transfer of the Cyt1Ab1 (cytolitic factor) and Cry11Bb1 (main mosquitocidal protein) genes from *Bt* subsp. medellin to toxic strains of *Bt*, specifically the acristalliferous mutants *Bt* subsp. israelensis 4Q2-81 and *Bt* subsp. *thuringiensis* SPL407, and the wild type strain *Bt* subsp. israelensis 1884; have been devoted to transfer the genes formerly mentioned to *B. sphaericus* strains.

First of all the genes were subcloned in a shuttle vector compatible with *B. sphaericus*, pMK3, it was obtained the constructs pMAR3 containing the gene Cry11Bb1 and pCytM containing the gene Cyt1Ab1. The last recombinant plasmid was introduced in the strain *B. sphaericus* 2297 which adquired the gene of the cytolitic protein of *Bt* subsp. medellin; it was the same case with the first construct wich was used to transform the strain 2362 of *B. sphaericus* lacking the modification-restriction system (restriction minus), this mutant was derived from the wild type strain *B. sphaericus* 2362. The first strain obtained by these manipulations was. tested against mosquito larvae including a line of *Culex quinquefasciatus* showing resistance to the native toxins of *B. sphaericus*, the other strain is under molecular characterization.

Since the lack of reproducibility and the frequent feilure of the transformation due to the modification-restriction systems of the wild type strains in the electroporation experiments, it was decided to use the conjugation as an alternative method to transfer the genes of the mosquitocidal proteins to *B. sphaericus*. According to this new approach, it was necessary to obtain the conjugative transference origin of the plasmid RK2 from *E. coli* clone into the vector pCTC1, which was provided by professor Fergus Priest, Heriot-Watt University, Edinburgh, Scotland.

The DNA fragment was subcloned into the recombinant plasmid pMAR3 generating the construct pMAR4 which is able to be transfered in trans using the mobilization functions of *E. coli* donor strains harbouring a helper plasmid derived of the RK2 system. The bacterial conjugation experiments are being conducted at the professor Priest laboratory in Edinburgh, along to the subcloning of the *Bt* subsp. medellin genes into the vector pCODA12, this plasmid is to be integrated in the bacterial chromosome of *B. sphaericus* looking for the highest stability of the recombinant strains to be produced.

GENETIC CHARACTERIZATION OF THE PATHOSYSTEM *Phytophthora infestans/Solanum tuberosum* AND ITS RELATIONSHIP WITH MOLECULAR POLYMORPHISMS

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OBJECTIVES

- Identication of *P. infestans* resistant strains to the systemic fungicide metalaxyl.
- Characterization of fungicide sensivity or resistance in *P. infestans* strains using isozyme and RFLP analysis.
- Establishment of *in vitro* potato clones previously selected for field resistance to *P. infestans*.
- Obtaining of monozoosporic cultures of *P. infestans* isolates representing several Colombian regions mainly the Eastern region of Antioquia.

SUMMARY

In Colombia 170.000 ha are planted with potatoes. Potato consumption per capita in Colombia is 67 Kg. and its production constitutes the main source of direct or indirect jobs for many people. Direct employment generates *150 working* days per ha given job to about 90.000 people in Colombia. Indirect employment is represented in transport, marketing and industrialization of potatoes.

From the 30 improved commercial varieties released by the Colombian Agriculture Institute (ICA) and the Universidad Nacional/ICA, only 6 are planted in Colombia: Parda Pastusa, Diacol Monserrate, Diacol Capiro, ICA Puracé, UNICA and Morita. The Diacol Capiro variety has a double use as a fresh potato and also in the processing industry. These factors have increased the demand of this variety in Colombia and in some processing industries of Venezuela and Ecuador.

Continuous planting of the Diacol Capiro has led to a genetic erosion of this variety, making it more susceptible to diseases. This phenomenon has contributed to increase the control cost of late blight of potato in different areas of Colombia, because 14 to 16 fungicide applications are required per cropping cycle.

In this research project we propose the collection, replication, characterization and storage of *P. infestans* strains.

For the characterization of *P. infestans* isolates we propose the use of physiological race, mating type and molecular analysis of isozymes and DNA analysis using RFLP and PCR techniques (RAPDs).

At the present time (1998) we have the following results:

- We have been able to standarize the methodology for *P. infestans* physiological race characterization. This methodology has allowed us to detect a big complexity in virulence factors present in the fungus. Results indicate that populations of *P. infestans*

present in extensive potato systems show a big homogenicity contransting with a big heterogencity present in those populations from mixed crop systems (Potato plus other Solanacea species such as tomato, water cucumber, bell pepper, physallis, and same wild species).

- For the molecular analysis of *P. infestans* isolates we have developed monozoosporic cultures using different methodologies.
- Isoenzyme analysis of *P. infestans* using glucose phosphate isomerase and peptidase have shown homogenicity among the isolates evaluated.
- A DNA extraction method has been standardized which has allowed us to do several evaluations such as:
- Chaff electrophoresis of *P. infestans* isolates of which four bands can be detected, indicating that *P. infestans* probably has four or more chromosomes.
- Preliminary results of DNA amplification by PCR using random primers (RAPDs) showed some polymorphism between isolates from different Solanacea species.
- We now are standardizing the RFLP technique to work with the RG57 probe.
- Using *in vitro* bioassays we have found several *P. infestans* isolates showing resistance to metalaxyl. These isolates will be used in the future for diagnostic purposes based on molecular techniques.
- The sexual stage of *P. infestans* has not been detected jet among the evaluated isolates only A1 mating type has been observed.
- A care collection of *P. infestans* has been initiated which now includes isolates from different Colombian potato growing areas, mainly from Antioquia. Now the collection has 85 isolates.
- The use of detached leaf inoculation method has been useful in detecting cross infections of *P. infestans* isolates on different Solanacea species. It's something we consider may constitute a potential inoculum source in the field.
- We have studied some biochemical mechanisms implicated on the potato defense against *P. infestans*. Preliminary results of some therpenic compounds synthesis induced in potatoes by some complex *P infestans* races, indicate a correlation between the levels of these compounds and the resistance present in the variety under study: tuquerreña variety (very susceptible), Diacol Capiro variety (susceptible) and UNICA variety (tolerant).

GENETIC ENGINEERING FOR CUCUMBER MOSAIC VIRUS (*Cucumovirus, CMV*) RESISTANCE IN *Musa spp.* COMMERCIAL SPECIES IN COLOMBIA

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OBJECTIVES

- Obtaining and use of an efficient system for banana transformation mediated by *Agrobacterium*.
- Evaluation and selection of *Agrobacterium tumefasciens* strains capable of transforming Colombian cultivarieties of banana and plantain, using a transformation vector containing Kanamycin gene NPTII as selection marker and antocynin gene as reporter gene.
- Evaluation of stability and hereditaribility of the reporter gene introduced to transgenic Musa species after several stages of clonal propagation and determination of regeneration frequency of chimerical plants and media to eliminate chimerism.
- Creation of transgenic banana plants using a transformation vector containing the proteic coat gene, CMV, regulated by promoter CaMV35S for evaluation to viral resistance under controlled conditions and field tests.
- Amplification and comparison of regions of genes codifying CMV coat protein virus, from various Colombian CMV isolations, using polymerase chain reaction.
- Creation of a cDNA library of CMV, for CMV coat protein genes isolated and cloned using as probe the fragments of gene amplified by PCR.
- Creation of an *Agrobacterium* transformation vector, containing Kanamycin gene NPTII as an adequate marker, and CMV coat protein virus.

SUMMARY

In vitro cultures of cultivars Dominico Hartón, Gros Michel, Grand Naine and Mallaccensis have been established, by using tissue culture techniques, for genetic improvement experiments.

Various experiments of transformation by particle bombing in embryogenic cells have been carried out in Dominico Hartón and Three Hand planty varieties. These two varieties came from the Catholic Univeristy of Louvaine. Today there are 47 containers with potentially transgenic plantain cells, bombed with plasmids containing the CMV coat protein gene of and the reporter gene Gus A. These cells are in the *in vitro* regeneration stage, and it is expected to obtain regenerated transgenic plants. The presence of the expected genes will be tested by PCR and by southern and northern blot the number of trangenes copies will be confirmed. Additionally, the expression of CMV coat protein will be confirmed first by using ELISA and then by western blot.

Simultaneously, transformation studies evaluating transitory expression of beta-glucoronidase in embryogenic cells using *Agrobacterium tumefasciens* have been undertaken. Up to now, the results cannot be compared to the results of biobalistics, but they show the possibility of applying the method in plantain cell suspensions.

Tobacco transgenic plants were obtained, and then transformed with CMV coat protein, belonging to the first offspring of the obtained the previous year. These plants were kept in the bio-security greenhouse specifically designed for working with this type of material. These plants will be challenged with Colombian CMV isolation for establishing the tolerance levels of plants to the infection, under greenhouse conditions.

In order to obtain the inoculum for the bioassay, healthy *Nicotiana tabacum* plants will be inoculated with an extract of an infected plant with Colombian CMV obtained at CIAT. Once the virus is purified, and its infectious potential determined, the bioassay will follow.

IDENTIFICATION AND CONTROL OF THE VIRUSES THAT AFFECT PASSION FRUIT (*Passiflora edulis*) IN COLOMBIA

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OBJECTIVES

- Characterization the viral complex that affects passion fruit in Colombia and develop diagnostic systems for its detection.

SUMMARY

Between 1996 and 1998, CORPOICA researchers surveyed the main passion fruit production areas in 11 departments of Colombia, where a total of 338 samples were collected from diseased passion fruit plants. The analysis of these samples was carried out at the CORPOICA Experiment Station and at the Virology Research Unit of the International Center for Tropical Agriculture (CIAT), Palmira, Valle. The samples were examined by electron microscopy, serology, using monoclonal and polyclonal antibodies, electrophoresis of viral nucleic acids, cDNA amplification using RT-PCR, cloning, hybridization and sequencing of viral nucleic acids. The survey and analyses showed that the phytosanitary situation of this important fruit crop in Colombia is manageable. To date, the prevalent virus is a *Potyvirus* transmitted by aphids. An isolate of this *Potyvirus* from Roldanillo, Valle, was cloned and partially sequenced, demonstrating a close nucleotide sequence similarity (97%) to a strain of soybean mosaic virus (SMV). This virus is different from the main *Potyvirus* infecting passion fruit in other countries around the world, (Passion fruit woodiness, PWV). The second virus detected so far in the departments of Valle, Antioquia and Santander, has been shown to be a member of the tymovirus group, serologically related to *Desmodium* yellow mottle *tymovirus* (DYMV). Tymoviruses may or may not have a vector (Coleoptera), but the passion fruit tymovirus isolated in Colombia has not been transmitted by either aphids or chrysomelids. These viruses can now be rapidly detected and diagnosed in reproductive passion fruit material, using serological methods. The *Potyvirus* can also be detected by molecular techniques, such as nucleic acid hybridization, using specific cDNA probes. The *Tymovirus* is being studied to develop a specific probe against a conserved region found in tymoviruses, called the "tymobox". The indexing methods available have made possible the production of virus-free, propagative material of commercial passion fruit varieties. Field experiments are being conducted to determine the degree of spatial isolation needed to avoid re-infection of virus-free planting stock in the main passion fruit growing region of Colombia, in the Valle del Cauca. The possibility of producing virus-free planting material of commercial passion fruit varieties, coincides with the need to increase passion fruit production in Colombia to meet the increasing internal and external demand for this fruit.

IN VITRO PROPAGATION AND MOLECULAR CHARACTERIZATION OF *Aniba perutilis* Hemsley AND *Musa acuminata* Collar, SELECTED AS PROMISSORY BY THE ALTO SAN JUAN COMMUNITY, RISARALDA

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OBJECTIVES

- Propagation by *in vitro* tissue culture of *Aniba perutilis* and *Musa acuminata*. Both species have been selected as promissory by the Alto San Juan community, in Risaralda.
- Genetic characterization of *Aniba perutilis* and *Musa acuminata*.
- Establishment and micropropagation by somatic embryogenesis of *Aniba perutilis* and *Musa acuminata*.
- Study of polymorphisms, applying molecular biology techniques and RAPDs, of *Aniba perutilis* and *Musa acuminata*.

SUMMARY

Aniba perutilis is a native species under strong human pressure due to its precious wood. *Musa acuminata* yields edible fruit, which forms the alimentary basis for the population of Alto San Juan (border between Risaralda and Chocó departments). The aim of this project is to undertake the massive propagation of these species by in vitro tissue culture, as well as their molecular characterization at a regional level, through RAPDs (Random Amplified Polymorphic DNA).

Musa acuminata

10 microbasins of San Juan river's tributaries were selected, and colino for *in vitro* multiplication was collected there. There is a protocol for massive *in vitro* multiplication of *Musa acuminata*, and a 5.300 plant *in vitro* stock in growth room.

Monthly, 100 plants are transferred into the CARDER greenhouse, near UTP, and there they are hardened under controlled conditions. After a month, they are taken to Santa Cecilia, Alto San Juan, Risaralda. The research center has established, together with CARDER, a demonstration plot for *Musa acuminata* plants, in order to evaluate their agriculture behavior and to foster peasants to grow the plant material produced in the laboratory.

A protocol for DNA extraction of *Musa* species has been developed, by extracting *Musa* DNA from 7 different sites.

During the research with RAPDs molecular markers, 60 Operon primers have been used. These primers were donated by CIAT, and 11 out of the 60 have shown polymorphisms that will lead to the species regional characterization.

Aniba perutilis

Trees selected in the Santa Cecilia zone, Alto San Juan, Risaralda, will be used to study the genetic diversity of the species. 20 trees from the Magdalena Medio region, acquired from Universidad Católica de Oriente, are kept in greenhouse and used as an explant source to develop a protocol for *in vitro* multiplication of the species. A protocol for *in vitro* establishment of nursery tree leaf tissue and for induction and proliferation of callus has been developed. Various culture media and hormonal concentrations are being evaluated to find the best to induce somatic embryo and/or bud production. There is a DNA extraction protocol (modified Dellaporta and clean up with activated coal) in conditions allowing its amplification.

INCIDENCE, DISTRIBUTION AND TYPES OF VIRAL STRAINS OF CITRUS TRISTEZA CLOSTEROVIRUS (CTV) IN A 350 ha IN ANTIOQUIA DEPARTMENT. COLLECTION AND ESTABLISHMENT OF MILD STRAINS OF CTV WITH POTENTIAL FOR USE IN CROSS-PROTECTION TESTS

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OBJECTIVES

- Establishment of incidence and distribution of the different strains of CTV (Citrus Tristeza *Closterovirus*), both severe and mild, in a 350 ha area in Antioquia department.
- Collection of mild CTV strains for using in cross-protection tests.
- Proposal of a pilot plan for phytosanitary certification of citrics.
- Molecular characterization of different CTV strains, both mild and severe, in the proposed area, by ELISA, immunoblotting, polyclonal and monoclonal antibodies, electrophoretic patterns of dsRNA and cDNA sequencing of the coat protein gene of a mild strain and a severe strain.
- Selection of local mild CTV strains (Mompox and Antioquia), through a biological evaluation for production of a mild chlorosis in the leaf nervures in plants as Mexican lime in greenhouse conditions.

SUMMARY

The mayor goal of this project is to establish the incidence and distribution of the different types severe or mild strains of CTV (Citrus Tristeza *Closterovirus*), in the regions of Antioquia and Llanos Orientales, by ELISA and the electrophoretic analysis of the double strand RNA in extract of infected tissue. The achievement of this aim will contribute to the analysis of molecular epidemiology of CTV, and the development of effective strategies of control of this citric pathogen, causing mayor economic losing in different countries. It would also be possible to select candidate strains to be used in cross protection and transgenic plants programs. Research in CTV control has been centered in the production of resistant or tolerant graft, but some CTV strains with the ability to infect plants growing on tolerant rootstock have been detected in some South American countries. Today cross-protection, the protection induced by mild strains of CTV against a severe strain, is one of the most effective methods for the control of CTV infection. Cross-protection has been used commercially in Brazil. It has been established however that no every mild CTV strain in able to induce cross protection, and therefore a serological and molecular characterization of candidate strains in a cross protection program is required.

INTEGRAL MANAGEMENT OF IN VITRO TISSUE CULTURE METHODS IN THE POTATO SEED PRODUCTION

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OBJECTIVES

- Development of a scheme for integral management of in vitro tissue culture methods for producing high quality potato seed under 3.000m above the level of the sea.
- Distribution of the in vitro multiplied plants to producers in CORPOICA zone 1, as high quality source material.
- Development of technology transfer towards the participative production of potato seed.
- Evaluation of the production of potato seed under 3.000m above the level of the sea, in order to determinate the feasibility of setting up a commercial program.
- Maintenance of a bank of free disease clones to produce seeds.
- Evaluation of the utility of using tubers produced in vitro in the production of seed within a farm.
- Reactivating of the use of diagnose media for viral diseases in monitoring seed in farms.

SUMMARY

The market for potato seed in Colombia is still low (1%) and the access to the potential market is restricted by both geographical and economical constraints. The use of producing zones under 3.000m above the level of the sea is an ecological need and an opportunity for non-exploited zones in potato seed production.

This project aims to integrate biotechnological methods that support seed production and genetic improvement by giving in vitro plants to producers and evaluating the production of high quality seed under 3.000m above the level of the sea.

ISOLATION AND CHARACTERIZATION OF INDIGENOUS *Bacillus thuringiensis* STRAIN COLLECTED IN DIFERENT PLACES OF COLOMBIA

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OBJECTIVES

- Determination of biopesticidal activity of 15 indigenous strains of *B. thuringiensis* against *S. frugiperda*, by bioassays.
- Identification of a promissory *Bt* indigenous strain, within the collection of the institute, with biopesticide activity against *S. frugiperda*, and evaluation of this activity in greenhouse bioassays, using a formulation prototype previously designed.
- Total or partial characterization of gene Cry X, present in IBUN 2.6 strain.
- Diagnose the biopesticidal activity of 15 *Bt* indigenous strains by PCR.
- Determination of biopesticidal activity of the same 15 *Bt* strains, by bioassays against first instar larvae of *S. frugiperda* insect. The activity will be expressed in terms of rate of mortality.
- Determination of CL50 of the strains with a higher mortality rate in relation to the pattern strain HD 137.
- Design of a formulation prototype of moisturizable powder from promissory indigenous strain.
- Evaluation of biopesticidal activity of promissory strain(s) in greenhouse according to laboratory results, using the obtained CL90.
- Partial sequencing of Cry X gene of IBUN 2.6 indigenous strain, by dideoxi method.

SUMMARY

The determination of Cry I genes in *Bt* indigenous strains was achieved by PCR, from analyzing polyacrilamide-SDS gels. The isolations presented the typical protein bands of 130 kDa, and 48% of the indigenous strains showed a protein profile around 60-70 kDa approximately. Sequences of six Cry genes were amplified: 1Aa, 1Ab, 1Ac, 1C, 1D, 1E. Seven PCR profiles different from cry1 genes were found in Bt isolations. 86% of these isolations contains more than one cry1 type gene.

The determination of biopesticidal activity was performed by the method of the diet superficial contamination. Bioassays took place on polystyrene sheets of 24 ponds in which 2 ml of artificial diet for breeding the insect were poured. After solidification, the substance is contaminated with 40 ul of the doses for evaluation. Then, each pond is infested with first instar *S. frugiperda* larvae. The activity is determined by using a diet of 130 ng in suspension per square centimeter of diet. 24 larvae are used for the treatment, and three repetitions of it. These bioassays are read every seven days, for counting the dead larvae per treatment.

Strains IBUN 2.6, IBUN 23.2 and IBUN 26.2 were selected as promissory, according to a mortality rate of more than 50%. Strain IBUN 2.6 showed the highest mortality rate in activity tests. The lethal concentration was determined as 50 and 90, by using protein

doses of 0, 30, 60, 90, 120, 150, 180 and 210 ng/cm2. 24 larvae were employed in three repetitions. The obtained data were processed with the statistics program PROBIT. The obtained values for IBUN 2.6 strain were 83.68 and 670.96 ng/cm2 respectively.

The design process of the prototype aimed to select preliminary the auxiliaries, evaluating them in order to define their components. Zinc oxide, titanium oxide, gelatin and tricalcium phosphate were selected preliminary as auxiliaries. Those were characterized according to parameters of rest angle, voluminousity (mg/g), humidity (%) and moisturizability (seg). During the elaboration of a moisturizable prototype, binary mixtures were prepared in various proportions, within a range of 50:50 to 90:10. The proportion with the best behavior in appearance, rest angle and voluminousity was selected. This prototype was the vehicle of the active ingredient of strain IBUN 2.6 that showed a highest activity against *S. frugiperda*.

Greenhouse tests were carried out within a system of plastic cover and walls with anti-afid net, with 4 1,5 m high concrete beds. For the assay, sweet corn plants planted in 5 lt plastic bags, in a mixture of rice peel, coal residue and soil were used. The plants were handled in agricultural terms according to the recommendations for a commercial corn crop. They were subject to the following conditions: rain and absence of rain, in order to evaluate the permanence of the product on the leaf; presence and absence of sunlight, in order to evaluate the effect of it on the product. The prototype subject to rain presented no decrease in the mortality rate, and sunlight does not affect the rate. Sequencing Cry X gene of the promissory strain showed that it is composed by 7 plasmids. PCR showed that it contains the genes Cry 1 (Aa, Ab, Ac, IC, 1D).

ISOLATION AND IDENTIFICATION OF BANANA RESISTANCE GENES AGAINST BLACK SIGATOKA

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OBJECTIVES

- To identify and isolate resistance genes against black Sigatoka (*Mycosphaerella fijiensis* Morelet) in banana cultivars of the Colombian Musaceae Collection.
- To standardize an inoculation/infection method for *Mycosphaerella fijiensis* for the banana varieties 'bocadillo comun' (genome AA) and Malaccensis type Pahang (genome AA), recognized as highly susceptible and resistant, respectively.
- To carry out a comparative cytological study of germination of the fungus and symptom development on the resistant and the susceptible cultivars inoculated with *M. fijiensis*.
- To isolate mRNA and make cDNA libraries from susceptible and resistant plants at different times after inoculation with *M. fijiensis* and before appearance of disease symptoms.
- To isolate unique clones induced by treatment of susceptible and resistant plants with the pathogen using subtractive hybridisation techniques.
- To study and compare the induction kinetics of mRNAs in susceptible and resistant cultivars during the infection process.
- To compare and select cDNA clones induced by the treatment exclusively in resistant plants using subtractive hybridisation.
- To identify the selected cDNA clones by partial sequencing and comparison to sequences reported in the gene bank.

SUMMARY

Banana and plantain are an essential part of the family basket in Colombia and their export constitutes an important factor in the generation of income for the national economy. Colombia ranks third in banana production worldwide, the cultivated area covers ca. 30.000 Ha. Plantain is cultivated on an area of 350.000 Ha distributed evenly all over the country, production volume exceeds 2.5 million tons per annuum, 86% for local consumption and the remainder for export.

Diseases caused by fungi, bacteria and viruses are one of the major limitations to productivity of banana and plantain. The most destructive foliar disease is black Sigatoka, caused by the pathogen *Mycosphaerella fijiensis*.

The use of non-conventional techniques, such as molecular biology and genetic engineering constitute a promising alternative for the generation of plants resistant to

these diseases. A whole arsenal of defense genes and naturally occurring substances are known to be induced in plants as a response to the attack by insects and/or pathogens. Molecular biology methods offer the possibility to clone and characterize genes related to defense and introduce them into commercial crops to render them genetically resistant.

The purpose of this work is to identify and isolate defense genes related to resistance against black Sigatoka (*Mycosphaerella fijiensis* Morelet) in the Colombian Musaceae Collection.

Up to date, plants of Malaccensis (Highly resistant), Baby banana (Susceptible), Gros Michel, and Gran Naine (Highly susceptible) have been introduced and multiplied by meristem-tip culture. The last two varieties were introduced because Baby banana can be tolerant under certain environmental conditions (R. Swennen, and J. Valencia, personal communication). These varieties have been transferred into soil to be used in the standardization of an inoculation method with *M. fjiensis*. It has been possible to establish and maintain a good quantity of monosporic cultures of the fungus from different regions of the country, which have been employed for the biological assays of infection of healthy plants.

While a biological assay is established, it has been possible to advance in the molecular experiments usingl resistant and susceptible plant materials from Musaceae Colombian Collection located at C.I. Tulenapa, Caribia, which is maintained under natural conditions and is permanently exposed to a high pressure of the pathogen. A method for the efficient isolation of RNAs was standardized. These RNAs were the starting materials to construct three cDNA libraries from the resistant genotypes Malaccensis, Balbisiana y Gran Naine (The last one was selected due to its tolerance against Black Sigatoka). Subtractive hybridization and RNA fingerprinting methods were used for the identification and isolation of differential cDNA sequences expressed as response of fungus infection in the resistant cultivar Malaccensis. RNA fingerprinting methods were more efficient than subtractive hybridization.

The knowledge derived from the infection modal will aloud to confirm through northern analysis the differential expression of genes isolated by RNA fingerprinting. The three-cDNA libraries prepared from resistant materials are gene reservoirs to contribute to the final goal of this work, that is the isolation of defense genes related to the resistance of black Sigatoka.

ISOZYME AND MOLECULAR CHARACTERIZATION OF PLANTAIN CLONES OF THE COLOMBIAN *Musaceae* COLLECTION

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OBJECTIVES

- Characterization of plantain clones of the Colombian Musaceae Collection using morphologic descriptors, biochemical and molecular markers.
- Evaluation of genetic diversity of the collection in order to identify potential duplicates and analyze the parameters of the species.
- Analysis the information generated through this study for its future use by growers.

SUMMARY

The family Musaceae is composed by two general groups: *Musa* and Ensete. *Musa* contains approximately 30 species including *Musa acuminata* and hybrids of *M. acuminata* and *Musa balbisiana*, which account for most of the edible bananas and plantains. Modern cultivated bananas are usually triploid, partenocarpic, and clonally propagated. Colombia has a great variety of edible plantains, of which only five are cultivated. The Colombian *Musa* Collection (CCM) has 130 accessions, although few of them have been characterized at the morphological and agronomic level. Genetic studies based on molecular agronomic and biochemical markers, will allow a better understanding of the CCM's genetic diversity. We used 23 isozymes, 32 RAPD primers and one AFLP primer combination to characterize the CCM.

The isozymes DIA, EST, GDH, MDH, ME, PRX, PGI, PGDH, PGM, RUB, SKDH were polymorphic and gave clear banding patterns. EST and DIA represented 48% of the total polymorphism. Different banding pattems were evident among accessions with eight RAPD primers. AFLPs (primers E-AAG+M-CAT) showed the greatest polymorphism among all markers. Up to 25% of the 166 scored bands were polymorphic. A similarity matrix based on the DICE coefficient of similarity Was produced and a dendrogram (UPGMA) was constructed using RAPDs or AFLPs. RAPDs barely differentiated the plantains (AAB and ABB) from the *acuminata* (AA and AAA), while AFLPs clearly separated plantains, bananas and wild accessions. AFLPs also showed differences within each

group as a result of a wider genetic diversity in bananas than in plantains. However, the Cavendish and Gros Michel genotypes were very similar to each other.

These results highlight the effectiveness of AFLP typing to identify CCM's accessions. It may speed up the designing of breeding strategies. Future activities will include surveying one additional AFLP primer combination (E-AAG+M-CTT) which, in preliminary assays, showed high polymorphism. Microsatellite primers, provided by CIRAD, will also be included in future surveys.

JUVENILE MATERIAL MICROPROPAGATION AND ADULT MATERIAL REVIGORIZATION ASSAYS OF FOREST SPECIES (*Decussocarpus rospigliosi* (Pilger) of Laub AND *(Quercus humboldtii*) Bondpland USABLE IN HYDROGRAPHICAL RIVER OF BOYACA

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OBJECTIVES

- Development of a procedure that permits micropropagation juvenile material, to be applied in massive multiplication programs of plant material usable in river basins.
- Evaluation of the micrografting technique as an alternative in order to induce revigorization of adult materials, to cloning selected trees.

SUMMARY

Due to the unbalance that the man has caused on the ecosystems, especially with the general chopping of trees, the inadequate utilization of land, the wrong utilization or lack of technologies for the adequate handling of the natural resources, mainly of the forests systems, river basins and their adjacent areas are subject to continuous deterioration, and therefore, the readiness of profitable water resources is every day scarcer. The conservation as well as the recovery of basins demand, the availability of vegetative materials of native species and or adapted to geographical specific areas.

Unfortunately most of the adequate species for these activities, and particularly the forests, present problems of natural generation, limiting their availability in their natural habitats drastically.

Decussocarpus rospigliosi and *Quercus humboldtii* are two common species of the river basins of Boyacá and they present problems of natural regeneration. Due to tree destruction before that they reach their sexual maturity, the production of seeds is being limited progressively. The importance of these species in the forests systems of basins prescribes the application of new technologies that permit their vegetative multiplication, avoiding the imposed restrictions for the sexual reproduction by seeds.

In order to produce vegetative material of forest species for the river basins, micropropagation is an alternative procedure utilized for vegetative propagation, development of massive multiplication programs and creation of orchards nurseries or vegetative propagation orchards of *D. rospigliosi* and *Quercus humboldtii*. Procedures and techniques for the development of this project will be based on processes of direct organogenesis in juvenile material and micrografting for adult material revigorization.

MOLECULAR MARKERS FOR CHARACTERIZATION OF *Rubus glaucus* AND TWO FORESTAL SPECIES: *Cordia alliodora* AND *Alnus acuminata*

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OBJECTIVES

- Development of genetic markers (RAPDs) for characterization of *Rubus glaucus*, *Cordia alliodora* and *Alnus acuminata* species.
- Study of genetic relations among 11 selected individuals, and comparison of the results with other natural populations identified in distant sites such as Santa Cecilia (border between Risaralda and Chocó departments).
- Characterization of *in vitro* propagated *Cordia alliodora* plantules, and determination of their clone correspondence by digital features.
- Development of RAPD technique for determination and genetic stabilization of *in vitro* propagated *Rubus glaucus* material.
- Development of RAPD technique for determination and genetic stabilization of in vitro propagated *Alnus acuminata* material, and evaluation of germplasm diversity from two different river basins.

SUMMARY

This study is the continuation of a project supported by COLCIENCIAS in 1994 (Selection and propagation of *Cordia alliodora* and massive propagation of *Rubus glaucus* and *Alnus acuminata*), which yielded the *in vitro* propagation of *C. alliodora* and *R. glaucus*, and the regeneration of *A. acuminata* by somatic embryogenesis.

The development of molecular markers (RAPDs) in *C. alliodora* aims to study the genetic relationships among 11 selected individuals. These relationships will be compared to other identified natural populations in distant sites such as Santa Cecilia (Risaralda). Then, the plants propagated *in vitro* will be characterized, and the clone they come from will be determined. There exists a methodology for regenerating *A. acuminata*, but it is necessary to develop a technique for certifying the genetic stability of *in vitro* propagated material. Then, the natural populations of this species will be compared molecularly in two river basins: Quindío and Otún river basins.

In order to guarantee the genetic stability of *in vitro* produced *R. glaucus* and the quality of clones it is necessary to develop molecular markers that allow, as RAPDs do, to do a follow-up from the subculture stage to the obtention of material in nurseries. Therefore the material that will be handed to the planter can be certified.

Cordia alliodora

There is a germplasm bank (*in vitro* germinated seed), collected in cooperation with CENICAFE. This research center collects the seeds from selected trees over the department and supplies themto the laboratory, after identifying the town and village where the seeds come from. The DNA of the seeds is extracted in the laboratory.

The DNA extraction protocol consists of an extraction buffer with CTAB and activated coal for cleaning up contaminants. There is a collection of genomic DNA of seeds from selected trees in five different origin sites, in good conditions for amplification.

60 Operon primers have been donated by CIAT (Tropical Agriculture International Center), and 18 of them have resulted polymorphic.

Rubus glaucus

There is a germplasm bank from 5 sites in the coffee-growing region, collected in cooperation with CORPOICA. This research center selects and sends the material to the laboratory, as its part of the inter-institutional agreement for massive propagation of *Rubus glaucus* between the Departmental Coffee-growers Committee, CORPOICA and UTP.

There are DNA extractions from random samples in various cycles of multiplication of the massive production process of *in vitro* plants, for the study of genetic stability of the species during in vitro culture and after 10 multiplication cycles. A DNA extraction protocol was developed, with a buffer with CTAB and activated coal for cleaning up contaminants. The DNA from 10 sites and from *in vitro* clones is in stock for its future analysis of genetic stability.

60 Operon primers have been evaluated, and 8 out of them are polymorphic.

Alnus acuminata

The places where samples of leaf material from adult trees will be collected are already located in the Otún and Quindío river basins.

There is a DNA extraction protocol for forestal material that uses a buffer with CTAB and activated coal for cleaning up contaminants.

There is an *A. acuminata* plantule population *in vitro* in the Quindío river basin, which has been regenerated from somatic embryogenesis, and it is now in nursery evaluation stage.

OBTAINING OF *Bacillus thuringiensis* INDIGENOUS STRAINS WITH BIOPESTICIDAL ACTIVITY AGAINST COLEOPTERA, AND EVALUATION OF USE ALTERNATIVES IN *Solanum tuberosum*

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OBJECTIVES

- Development of a collection of indigenous isolations of *Bacillus thuringiensis* (*Bt*) and *Pseudomonas spp*, from various agroecosystems dedicated to potato planting.
- Characterization of *Bt* indigenous strains taking into account the biopesticidal activity (bioassays against *Premnotripex vorax*), after molecular and microbiological analysis.
- Colonization tests of the isolated *Pseudomona spp* on potato plants.

SUMMARY

Premnotripex vorax is one of the major pests of potato plantations in Colombia. Chemical pesticides are used for controlling this pest, in huge amounts (13.5 kg/ha/year), thus generating a strong environmental impact in terms of toxic effect on no-target organisms and the residual effect that contaminates the water-bearing layer. At the same time, it causes resistance problems, due to the indiscriminate use of the chemicals.

From soil samples taken at ecosystems with and without potato plantations, *Bt* indigenous strains presenting CryIII genes were isolated. 24 isolations were made, from 7 soil samples, and none of it contained CryIII genes.

The conditions for generating the breeding of *P. vorax* Hustachey were studied. The insect's life cycle was analyzed under manipulation conditions of the offspring: temperature (15°C) and humidity (70% RH). The various stages of the insect were analyzed, egg, larva, pupa, and it was concluded that the life cycle of *P. vorax* has an average duration of 96.7 days. In adult stage, the longevity can reach as long as one year.

Bioassays of *Bt* strains against *P. vorax* larvae, using Cry3B and Cry3D as controls, did not show a significant percentage compared to the absolute tester.

For the collection of Pseudomonas, isolations 45 samples from 2 places of Boyacá and 3 places of Cundinamarca were collected. The selection standards of plantations were temperature and precipitation, topography and humidity percentage, potato variety and fertilizer used. 212 strains with positive fluorescence in medium King B were selected, for undertaking chemical tests of negative Gram bacilli, produced by bioMerieux api 20 en. The results show that in all the analyzed potato producing zones there is a good association between the potato root system and fluorescent Pseudomonas. This fact confirms that *P. fluorescens* indigenous isolations can become a good host for plasmids bearing cry genes with pesticidal activity against soil pests in potato plantations.

PLANTAIN IMPROVEMENT THROUGH MUTATION INDUCTION FOR OBTAINING RESISTANCE TO YELLOW AND BLACK SIGATOKA

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OBJECTIVES

- Elaboration and development of protocols for standardization of in vitro methodology and speeding up of plantain meristem propagation.
- Production of discreet genetic changes in the original genotype for selecting new plantain varieties resistant or tolerant to black and/or yellow sigatoka and other pathologic problems such as lateriosis and nematodes.
- Field evaluation of the response of irradiated material to the above mentioned pathologic problems.
- Selection of tolerant and/or resistant material to the phytopathologic agents subject to evaluation.
- Facilitation of selection, description and characterization of the obtained promissory mutants by modern biotechnological techniques (RFLP, RAPD and PCR).

SUMMARY

Yellow sigatoka (*Mycosphaerella musicola*) and black sigatoka (*Mycosphaerella fijiensis* var Diformis) generate the major pathologic problems for banana and plantain production in the world. In Colombia, both diseases cause a loss in productivity of 50% and more.

The triploid condition of the widely cultivated plantain forms (*Musa sp* AAB Simmonds), added to the scarce variability registered in terms of response to yellow and black sigatoka have made difficult the obtaining of cultivars with a satisfactory response to these pathogens. That's why it is so imperative to establish alternative strategies as the proposals of this project for induced mutations, as an effective step for the genetic improvement of *Musa* AAA species.

This project is taking place in Departamento del Tolima, along the road from Fresno to Mariquita, vereda La Ceiba, at 1.250 m above the level of the sea, with average temperatures between 18 and 25 C, and 65-100% relative humidity, and 1.800 mm annual precipitation. 1490 Dominico Hartón plants treated with 60Co and 189 radiosensibilized plant have been evaluated.

The characterization of mutants have been achieved, and a relationship between the individual's size and leaf number was found, according to the mutation and degeneration systems of the various cultivarieties. During growth and development analysis 7 clones have been selected as promissory, as they bear the evidence of the effect of somaclonal

variation caused by the action of 60Co. The frequency of spore population of *Paracercospora fijiensis* and *Pseudocercospora musae* has been studied too. The results showed that in non-irradiated Dominico Hartón *P. fijiensis* prevails over *P. musae*. In relation to the incidence of aqueous rotting of Pseudostem, 58 clones irradiated with 60 Cobalt 25 Gray were evaluated, as well as 5 controls of the same material. 3 clones and controls 5 showed a good response to the disease.

The population dynamics of nematodes show behavior differences in different materials to *Melodoigyne* and *Helicotylenchus* genders.

PRODUCTION OF THE WASP *Cephalonomia stephanoderis* FOR BIOLOGICAL CONTROL OF COFFEE BERRY BORER

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OBJECTIVES

- To implement and improve the methodology developed by Cenicafe for the production of *Cephalonomia stephanoderis*.
- To produce 15 million wasps (*Cephalonomia stephanoderis*) a month.

SUMMARY

It is estimated that from the 1.1 million hectares where coffee is presently grown, 250 to 300 thousand hectares located below an altitude of 1.300 m will disappear in the near future, as has been happening in some Colombian municipalities, and the remaining 800 thousand will have to live with the coffee berry borer for the time to come. According to the National Coffee Federation, the speed of dispersal of the berry borer went from 10% of the coffee area in 1992, to 30% in 1994, and it topped 100% in 1995.

In view of this situation it becomes necessary to complement cultural practices incentivated by the Federation (the so-called RE-RE, Spanish for check, harvest, check over and re-harvest) with the benefits stemming from the production and utilization of the wasp *Cephalonomia stephanoderis* as a biological control of the coffee berry borer.

Considering the impact on productivity and quality of the grain by the berry borer and its dispersal, the implementation of biological control measures becomes a necessity.

The project is geared toward the improvement of the methodology developed by CENICAFE for the production of *Cephalonomia stephanoderis* (a coffee berry borer parasitic wasp) and to produce 15 million wasps a month.

Mass production of the wasp has already started and the first 600.000 wasps have been delivered to the National Coffee Federation.

STANDARDIZATION OF METHODS FOR CLEANING AND IN VITRO PROPAGATION OF PROMISSORY VARIETIES OF WINE GRAPE AND DESSERT GRAPE IN VALLE DEL CAUCA DEPARTMENT

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OBJETIVES

- Standardization and/or adaptation of methods for cleaning and quick *in vitro* propagation of free pest and disease material of grape varieties for dessert and wine identified by CENIUVA as promissory for the central and northern region of Valle del Cauca.
- Development and/or adaptation of cleaning techniques (thermotherapy, disinfection) of dessert and wine plant material, identified with pathogens, especially virus.
- Development and/or adaptation of protocols for *in vitro* tissue culture, leading to micropropagation of homogeneous, both fenotypically and genotypically, and healthy material of these grape varieties.
- Joint development of a methodology for transfer and distribution, together with growers, institutions or cooperatives, among growers in northern Valle del Cauca.

SUMMARY

Amongst the fruit grown in Colombia, grape shows a promising future both in national and international markets. Dessert grape has a stable demand throughout the year. The demand for fresh grape (85%) is supplied partially by domestic production (50%), and the remaining percentage by imports from Chile and USA. Almost 15% of domestic production are used for wine, but not all the varieties used for this are the adequate ones for wine.

The grape crop shows problems affecting production and competition. According to CENIUVA, almost 100% of the vines grown in the country are contaminated by virus as Corky Bark, Grapevine Leafroll and Grapevine Fanleaf Nepovirus. The contamination by these viruses can reduce production in a 50%. Additionally, bad agricultural practices may increase production costs for more than 40%, and enhance phytosanitary problems. This project aims to generate free virus plants that lead to an improvement in production and profitability of the crop.

The varieties selected as promissory are: *Vitis vinifera*, Queen and Italia varieties, as dessert grape; *Vitis labrusca*, Isabella variety, as dessert grape; *Vitis rupestris*, as grafting pattern.

Standardization of cleaning techniques, thermotherapy and disinfection, was achieved working on mother plants of experimental varieties. 60% of the buds of thermotherapy treated plants showed negative reaction to ELISA test with various antiserums. Mother plants without thermotherapy treatment showed a positive reaction to GLRV and GVA,

detected by ELISA. For standardizing the media for establishment, multiplication and rooting, meristems and axilar and apical tems of treated virus free plants were taken.

The parameters for selecting establishing media were budding rate, oxidation and death rate, latency rate, callogenesis rate and callo size record. A reading was done 15 and 30 days afterplanting. As multiplication medium, M&S and WPM media were used, with 5 BAP levels, evaluating rebudding rate, and oxidation and death rate. For evaluation of rooting media, independent tests were carried out, using as tester the previously selected medium. The standardization of this medium was based on rooting rates and characteristics of roots, and plant characteristics such as an increase on length and strength.

The statistical analysis of establishment and multiplication stages was based on Fischer tests in factorial arrangements distributed in random blocks. The results showed significant differences in budding rate, with an error margin of 5%.

Additionally, the standardization of a methodology for evaluating the acclimatization in greenhouse was completed. In vitro produced material, and then hardened in greenhouse, was planted in two experimental plots in Ginebra (Valle). 200 plants were planted, with a density of 1300 plants/ha. After 12 months, the in vitro plants showed a similar behavior to plants planted from cutting.

II. ENVIRONMENTAL BIOTECHNOLOGY

IMMOBILIZATION OF BACTERIA AND MICROALGAE IN ALGINATE BEADS TO REMOVE INORGANIC NITROGEN FROM AGROINDUSTRIAL WASTE WATER

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OBJECTIVES

- Evaluation of inorganic nitrogen removal from agroindustrial waste water, using a microalga-bacteria co-culture, immobilized in alginate beads.
- To determine the capacity of *Azospirillum brasilense* for microaerophylic nitrate reduction.
- Determination of the nitrifier competence of *Chlorella vulgaris* under microaerophylic conditions.
- Comparison of the effectiveness of differents types of bacteria/microalga co-cultures for removal of inorganic nitrogen from waste water.

SUMMARY

Microalgae cultures as tertiary waste water treatment offer big advantages compared to the conventional treatment processes, because they do not produce secondary polution when the biomass is harvested and they allow an efficient recycling of nutrients. However, one drawback of the microalgae cultures in suspension is how to recover the biomass produced once the treatment is over. To solve such problem, diferent systems of immobilized microalgae have been developed, using different supports like carrageenan, chitosan, alginate, agar and polyuretane, among others.

As result of a previous work, we found that the Chlorophylaceae *Chlorella vulgaris* displays efficiencies of ammonia removal of 97%. However, due to the nitrification process that occur in the microalgae cultures, the concentration of nitrates increases significantly (up to 600%).

Considering the difficulty of harvesting the microalgae biomass in suspension cultures, and the need for eliminating inorganic nitrogen from waste water in a more efficient way, this project intends to evaluate the joint activity of the microalgae *Chlorella vulgaris* and the bacteria *Azospirillum brasilense* immobilized in alginate beads. The first one oxides the ammonium to nitrates, and the second one, has the ability to reduce nitrates to gas nitrogen under anaerobic or microaerophylic conditions.

OPTIMIZATION OF STARTING STAGE IN ANAEROBIC REACTORS, BY IMPROVING THE QUALITY OF DIFFERENT SEED IN DYNAMIC OPERATING CONDITIONS

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OBJECTIVES

- Collection of an inventory of possible seed sources for starting up anaerobic reactors in Valle del Cauca and Antioquia regions.
- Determination of the effect of temperature on the behavior of reactor inocules, taking into account the climate difference between the two regions involved in the project (Cali, average temperature: 27 C; Medellín, average temperature: 20 C).
- Control of varieties as ascentional speed, organic carry, buffer capacity of the system, biogas production, AGV, pH and temperature, involved in the operation of anaerobic reactors.
- Determination of optimal relation between mud density and its specific methanegenic activity.
- Characterization and correlation of microbial population (biomass) according to the different stages that occur in an anaerobic reactor.

SUMMARY

Although anaerobic wastewater treatment processes are technically and economically feasible, they are not widely used due to, amongst other reasons, the starting up stage of these reactors. This stage has become a critical one, as there is not much knowledge about the relation with environmental and operational factors that work together and create an adequate, or non-adequate, medium for the normal development of anaerobic ecosystems.

On the basis of controlling all the variables involved in the development of biomass, the project aims to develop a methodology that allows optimizing the growth of viable anaerobic bacteria that guarantee a starting up stage within a short period of time. This will lead to a quick accomplishing a high level of efficiency in organic matter removal and biogas production.

III. HEALTH BIOTECHNOLOGY

DEVELOPMENT AND COMMERCIALIZATION OF AN HLA-DR TYPING SYSTEM BASED ON THE PCR-SSP METHOD

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OBJECTIVES

- To develop an HLA-DR typing system based on PCR-SSP methodology. This system will have a degree of resolution compatible with Kidney-transplant needs.
- Give technical assistance and employee training and for the laboratories that implement the new technique, as a replacement of serological methods.

SUMMARY

The identification of the polymorphism's at the loci of the Major Histocompatibility Complex (MHC) is of great importance in transplantation, specially in kidney and bonemarrow transplantation. There are many methods of HLA typing, but for definition the ideal technique must be fast because the results must be obtained within the time of coldisquemia, and must be accurate because the success of the transplant depends in great measure on the histocompatibility between donor and recipient. Since 1991, it has been shown that serology, the traditional methodology, has an error rate superior to 25%. It is for this reason that serology for Class II typing has been replaced by the new methods based on DNA typing in the majority of HLA typing labs in Europe and USA. In Colombia, however, serology is still the method of HLA-DR typing, even, with a higher inaccuracy, that diminishes the success of the transplant.

This project will develop a commercial system for HLA-DR typing based on the PCR-SSP technology. This new technology is by far more accurate than serology, and it is also less expensive, making it superior form the point of view of cost-benefit for the patients and laboratories. Although similar systems are on the market in USA and Europe, ours will have the advantage of being able to offer complete technical support to all the laboratories implementing the technique.

DEVELOPMENT OF DIAGNOSTIC TESTS FOR HORMONES (TSH hCG) BASED ON MONOCLONAL ANTIBODIES

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OBJECTIVES

- Reinforce the area of development of products based in the technology of monoclonal antibodies.
- To generate Moabs specific to Thyroid stimulating hormone TSH an Human Chorionic Gonadotrophin.
- Develope a prototype diagnostic kits for hormones.
- Develope a screening test for TSH that useful in detection programmes for early detection of Congenital Hypothiroidism.
- Develope a Pregnancy Home Test.
- Medium scale production d these two kits in order to offer a product which in competitive in quality and price with others available in the market.

SUMMARY

The major purpose of biotechnology is the generation of products that will allow Colombian Industry to be competitive in Colombia and abroad. Diagnostic products based in monoclonal Antibody Technology suffice these premises by far. A good evidence for this is the first stage of this product in which our company developed and scaled the production of ABO Blood grouping Reagents Based in MoABS for the first time. These products are now available in Colombian diagnostic market and have gained good acceptance.

In the current project our goal is the development of Diagnostic Kits for clinically important hormones such as hCG and TSH useful in the diagnosis of pregnancy and Congenital Hypothiroidim respectively.

This last disease constitutes a public health problem since it represents one of the major preventable mental retardation diseases. Our goal is to have in the near future a screening test for newborn that can be applied In our country as it has been done for some years in other countries.

MOLECULAR VARIATION IN INDIVIDUALS AND CRIMINALISTIC UTILITY IN COLOMBIA

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OBJETIVES

- Description of allelic and genotypic frequencies of eight systems in Colombian population to individual identification purposes.
- Determination of existence of variants that may not have been described in other populations around the world.

SUMMARY

Historically there are records about the genetic mixture of today people from Colombia. The word mestizo describes a king of variability which is extends to fauna and flora from our country. In Colombia the application of Molecular Biology in Criminalistic has appeared recently (three years ago).

This project wants to find out how the heterocigocity of alelic frecuencies is in the following systems; HUMvWA, HUMTH01, AMPLIFLP DIS80. DIS7, D4S139, D5S110, D8S358, and D17S79. With this results we intend to make a right interpretation of genetic tipyfications of blood stains, semen stains and hair roots, which are the most common kind of evidence in criminalistics cases. Through the development of the project it would be possible to know the similarly and differences between Colombian population and the other populations described around the world; besides, it is important for government institutions to get extensive knowledge in different methodologies (STRs, AMPLIFLP, and VNTRs) to manipulate adequately criminalistic evidences in the Colombian legal system.

RECOMBINANT ALERGENS FOR POTENTIAL USE IN ASTHMA: FUNCTIONAL AND MOLECULAR ANALYSIS

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OBJECTIVES

- Definition, by genetic manipulation and use of monoclonals, of the potential utility of recombinants Bt1 and Bt11a as reactives for treating allergic asthma.
- Production of alergens Bt1 and Bt11a, by using highly efficient expression systems in *E. coli* and yeasts.
- Standardization of skin tests and ELISA tests with recombinant allergens Bt1 and Bt11a.
- Production and characterization of monoclonal antibodies (against Bt1 and Bt11a).
- Identification of B epitopes of recombinants Bt1 and Bt11a.

SUMMARY

Recombinant allergens Bt1 and Bt11a (previously obtained by this research group) are two major allergens of *B. tropicalis*, with established DNA sequences and some immunological properties. As these allergens are equivalent to allergens contained in B. tropicales native extract, it is important to use them for research in order to define their potential as reactives for diagnose and treatment. The research includes efficient expression of recombinants, reproducing the characteristics of the native one; B and T epitopes determination by antibodies and monoclonal T lymphocytes; manipulation by mutagenesis of these epitopes in order to detect critical residues; determination of immunogenetic relationships (TCR and HLA) of response IgE on these allergens; determination of their utility as diagnose reactives and evaluation of allergens environmental level. This project seeks to define the use of recombinants Bt1 and Bt11a for diagnosing and treating allergic asthma.

IV. INDUSTRIAL BIOTECHNOLOGY

EXTRACTIVE BIOCONVERSION FOR THE PRODUCTION OF MUCOR *Miehei's* RENNET

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OBJECTIVES

- To combine an ultrafiltration step with a continuous production process of the enzyme renine from the zygomycete *Mucor miehei*.
- To determine the efficiency of separation of sub-products responsible for enzyme repression without affecting the productivity.
- To examine the possibility of improving productivity of the process and to quantify the effect of combining the filtration unit with the enzyme producing fermentor.
- To determine which aminoacids are responsible for repression in the production of the enzyme.

SUMMARY

The zygomycete *Mucor miehei* produces an acidic protease which is subject to feedback repression by fermentation sub-products. There are two options to overcome this physiological constraint; the first one is to combine the production and recovery steps to improve the overall productivity of the process, and the other one consists of applying the so-called extractive bioconversion technology.

The objective of this project is to combine a process of separation by membranes, such as ultrafiltration, with a process of continuous production of renine using a membrane bioreactor; when such a combination allows to couple production and recovery in one single step, it is called extractive bioconversion.

PRODUCTION OF THE SURFACE ANTIGEN OF THE HEPATITIS B VIRUS IN YEASTS USING RECOMBINANT DNA TECHNOLOGY

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OBJECTIVES

To develop a methodology for the obtention of the proteinaceous capsid of hepatitis B virus using *Saccharomyces cerevisiae* fermentation.

SUMMARY

Hepatitis B is a serious and growing health problem worldwide. The numbers published by WHO are worrisome: more than 2 billion persons have been infected, almost 300 million are chronic carriers and 2 million people die every year victims of this disease. Infectivity of hepatitis B is 100 times higher than HIV, the causative agent of AIDS.

This virus is highly infectious and commonly associated to liver cancer (hepatocarcinome), cirrhosis and chronic hepatitis. Only small inocula are needed to initiate the process of infection and the subsequent development of the disease, which is lethal in most cases. The virus has been detected in semen, saliva, urine, faeces, bilis, menstrual blood, vaginal secretions and pleural fluid.

The highest risk groups to contract hepatitis B are, health professionals, family contact with sick individuals, homosexuals, prostitutes, drug addicts, travellers to endemic zones, patients that have undergone renal transplants and hemodialysis, residents and personnel of psychiatric institutions.

In our country, inspite of the efforts undertaken by various institutions in the production of vaccines, there is still the need to develop production processes for the elaboration of biomolecules (monoclonal antibodies, recombinant proteins) for biomedical, alimentary, pharmaceutical and/or industrial use.

In the case of the vaccine against hepatitis B, a fragment of the viral capsid called the surface antigen (HBsAg) is used. Production of the antigen is controlled by a gene that is introduced into a yeast strain using a recombinant plasmid that has been previously amplified in *E. coli*.

The purpose of this project is to produce the surface antigen of the hepatitis B virus using recombinant microorganisms. Once we have obtained recombinant strains of *S. cerevisiae* and other yeasts we will evaluate the production of the viral protein in every one of them and study the environmental conditions that affect production.

PURIFICATION AND STUDY OF THE PROPERTIES OF PEPTIDASES FROM *Paralithodes camtschatica* HEPATOPANCREAS

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OBJECTIVES

- Purification and characterization of peptidases from the preparation Crab Colagenase.
- Evaluation of molecular properties of crab pure peptidases: molecular mass, isoelectric point, aminoacid and carbohydrate composition.
- Study of catalytic properties of pure peptidases, their optimal conditions for maximum enzymatic activity and substratic specificity.
- Evaluation of stability of crab peptidases under various denaturalizing conditions.

SUMMARY

There has been a considerable increase in the utilization of enzymatic preparations in various branches of industry, alimentary, textile, medicinal, perfumes and veterinary. Proteases, celulases and lipases are the most used enzymes (60% of the worldwide market of these products), but peptidases, oxidases and phosphatases are used too.

Artyukov and Sakharov isolated and extracted a multienzymatic complex from the crab Paralithodes camtschatica hepatopancreas, and named it Crab Colagenase (CC). The initial studies showed that this preparation contains at least 3 proteases with catalytic activities, which characteristics have already been determined. Spectrophotometric and chromatographic methods were used for detecting peptidases in CC preparation, using the following substances as substrates: Hipuril-L-phenilalanine (Hip-L-Phe), Hipuril-L-Arginine (Hip-L-Arg), Hipuril-L-lisine (Hip-L-Lis), L-alanine paranitroanilide (L-Ala-pNa) and L-Leucine paranitroanilide (L-Leu-pNa). The results showed that carboxipeptidases are very active, while aminopeptidases are not very active.

Purification of crab carboxipeptidase was done by low-pressure liquid chromatography. In the first place, ionic exchange chromatography was used, testing 3 supports: DEAE-Toyopearl; DEAE-Sepharose; DEAE-celulose). DEAE-celulose yielded the best results. The sample from ionic exchange chromatography was then under hydrophobic chromatography on Phenil-Sepharose, and the resulting carboxipeptidases showed a high degree of purity. In the final phase, gel-filtering chromatography was used, on Sephadex G-75 and Sephacril S 100. Both supports were efficient for purification, but Sephadex g-75 yielded better results.

The characterization of carboxipeptidases was achieved by electrophoresis in 12.5% polyacrylamide, with SDS and 2-mercaptoetanol. The molecular mass of the preparation was 40.000 Da, the optimal pH for activity was 7.5, and the dependence of activity on NaCl concentration was proved. Carboxipeptidase maximum activity was reached in 1 NaCl concentration.

Once the preparation was pure, the thermal stability was tested and the results showed that it is inactivated at room temperature, and it is stable at 5°C.

SCALE-UP OF THE ANTI-TETANUS VACCINE PRODUCTION PROCESS

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OBJECTIVES

- Establishment of an anti-tetanus vaccine production process, at industrial level.
- Scaling of the production process of tetanus toxin from *Clostridium tetani* fermentation.
- Selection of unitary operations for culture media pre-treatment and for tetanus toxin purification.
- Establishment of operative conditions of the mentioned stages.

SUMMARY

Clostridium tetani is the causing agent of tetanus, a lethal disease produced by the contamination of wounds with the microorganism spores. The spores germinate and produce a powerful toxin (tetanus toxin), the direct cause of the disease.

The disease can be prevented by vaccination, with the mitigated antigen (toxoid) supplied intramuscularly. Thus, the production of neutralizing antibodies against the toxin is activated. For obtaining the antigen, it is necessary to cultivate the microorganism, and extend it to the filtrates at the end of the autolysis phase.

For producing the toxin, *Cl. tetani* is cultivated at 35°C under anaerobiosis, in an enriched medium and with neutral pH, it is not proteolytic and the glucidolytic activity depends on the strain. Energy requirements are satisfied by a direct aminoacids reduction such as glucotamic, aspartic and histidin into CO_2, NH_3 and acetic and butyric acid.

Since 1940s the microorganism has been studied, with the aim of obtaining an adequate culture medium for its growth and toxin production. The medium used today is the Latham-Mueller. Several culture systems have also been studied: in lotes, continuous, semi-continuous, with or without agitation. The National Institute of Health (INS) uses the following culture system: submerged, without agitation, in 30 L glass jars, with 10 L of culture each. Each week, 6 jars are cultivated simultaneously, and they are taken manually to the autoclave. Then they are cooled down by immersion, and taken to the incubation room at 35°C.

The INS produces a considerable variety of biological products for human use. With these products, it covers wide immunization programs and other needs of the country's health sector. One of the most important products is the anti-tetanus vaccine. Demand for it in Colombia in 1996 was 13,5 million doses. The INS produced 9 million, and had to import the rest.

During the vaccine's production, the most important phase is the obtaining of the specific toxin by fermentation of *Cl. tetani*. The toxin, after several operations as inactivation, purification and adsortion, becomes a non-toxic product of high antigenic

power, and constitutes the basis of the vaccine.

For an easier control of the process, it is necessary to cultivate the microorganism in a fermentator which production level is enough for the required demand, which has the adequate systems for automatic control of the important variables, and which has a connection with the pre- and post-fermentation equipment. These conditions would prevent the manipulation of material during the process, reducing the biologic risk, and they would be a great step ahead to reach the WHO quality standards, as stated in the booklet Good Manufacturing Practices.

Up to now, the operative conditions of the fermentator have been determined, based on scale-up criteria: power per capacity unit and height/diameter proportion. Experimental information has been obtained for laboratory culture (5 liters) and the procedure for preparing the culture medium required for tetanus toxin production.

STUDY OF A FRUCTOSILTRANSFERASE FROM *Aspergillus niger* IN FRUCTOOLIGOSACARIDS SYNTHESIS

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OBJECTIVES

- Study of fructooligosacarids production from sacarosa, using fructosiltransferase enzyme from *Aspergillus niger*.

SUMMARY

Frutooligosacarids (neosacarids) are sacarose oligomers with fructose units joined by glicosidic links (b-2-1). They are resistant to digestion by mammal alfa-amilase, sacarose and maltase, therefore they are not digestible for humans. But they can be employed per Gram positive organisms, such as bifidobacteria. This activity allows the growth of intestinal flora, preventing constipation, increasing lipids in the blood stream, suppressing the production of putrefactive substances, and reduces toxic metabolites, harmful enzymes and prevents pathogenic diarrhea.

This project seeks to produce fructosiltranferase enzyme form *Aspergillus niger* by fermentation, in order to study it within the oligosacarids synthesis reaction. Various methods for purification of the enzyme will be studied, as well as the reaction conditions using sacarose as substrate, in order to optimize the production of fructooligosacarids.

V. LIVESTOCK BIOTECHNOLOGY

APPLICATION OF ANIMAL BIOTECHNOLOGY FOR THE PRESERVATION AND PROPAGATION OF COLOMBIAN CRIOLLO CATTLE

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OBJECTIVES

- To develop adequate methodologies for the obtaining of a large number of criollo cattle embryos at pre-implantation level.
- To cryopreserve 300 to 500 embryos of each of the seven criollo cattle races (Blanco Orejinegro, Casanare, Chino Santandereano, Costeño con Cuernos, Hartón del Valle, Romosinuano and San Martinero).

SUMMARY

The criollo cattle races in Colombia have gone through an adaptation process to the tropical conditions of the country. In the process of natural genetic selection desirable phenotypes have been generated, such as resistance/tolerance to internal and external parasites, the ability to tolerate high temperatures and humidity, the ability to strive on low quality forages, high reproductive efficiency and resistance or immunity against some diseases. Regardless, this genetic material has been vanishing as a consequence of the arrival of races bred for productivity -but not for adaptation to our medium- being almost at the risk of extinction.

The purpose of this project is to evaluate the response of the seven criollo cattle races with respect to embryonary biotechnologies, such as induction of superovulation, cryoconservation of embryos and *in vitro* fertilization, as a means to preserve and multiply this unique genetic resource.

It is known that one of the sources of variation in the embryo transfer programs is the bread and it is closely related to the dose of FSH utilized to induce superovulation. No reports are available about the superovolatory response of the Colombian Creole cattle breeds. Therefore, an experiment was carried out in Colombia to evaluate the response of these breeds to two different doses of Folltropin (FSH-p Vetrepharm - Canada Inc.-). The doses utilized were 24 mg as low (L) dose and 36 mg of FSHp as high (H) dose, the two doses were used with two daily injections with decreasing dose, during four days. Seven breeds were used to evaluate the response to the treatment. The response was measured by the number of CL palpated, the number of embryos collected and the quality of embryos collected. Transferable embryos, those with more than 70% viable cells. Results for each one of treatments and for each bread are shown in the following table.

Average superovulatory response of seven Colombian Creole breeds superovulated with two doses of FSH (Folltropin).

Breed	No. Obs.		No. CL		Number of embryos			
					Collected		Transferable	
	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo
Romo	10	5	6.2±1.0	5.6±1.1	2.6±0.7^{ab}	3.0±1.5^{ab}	1.2±0.5^{ab}	1.6±0.9^{ab}
BON	10	5	5.4±1.0	5.8±1.3	4.1±1.2^{a}	5.4±1.5^{a}	1.0±0.4^{ab}	1.6±0.6^{ab}
San Mart.	10	5	5.7±1.5	3.6±2.3	2.3±1.0^{ab}	1.0±0.8^{ab}	0.7± 0.4^{b}	0.8±0.6^{ab}
CCC	10	4	3.3±0.8	2.8±1.6	0.9±0.5^{b}	0.0±0.0^{b}	0.8±0.4^{ab}	0.0±0.0^{b}
Casanare	8	4	6.5±1.3	6.0±2.3	4.1±1.2^{a}	5.0±2.2^{a}	1.0±0.6^{ab}	2.0±0.9^{ab}
Hartón	6	5	5.5±1.7	7.4±2.5	3.5±1.7^{ab}	4.8±3.2^{a}	2.3±1.2^{a}	3.0±2.3^{a}
Chino	7	1	6.8±1.3	6.0	4.8±1.4^{a}	30^{ab}	2.8±1.1^{a}	10^{ab}

Data in the some column with different superscript are different. ($P<O.10$)

Statistiscal analysis was run with the Statistix® program, for a complete randomly design. The ANOVA showed differences between breeds for the number of embryos collected, with the lowest response for CCC and no statistical differences among other breeds. For the number of transferable embryos, newly CCC had the lowest response and the best response for Harton del Valle (although not statistically different from the other breeds), confirming that as it is reported in the literature, breed is another cause of variation in superovulatory treatments. There was no significant ($p<O.10$) differences between the two doses of Folltropin for any one of the parameters evaluated. No statistical interactions between bread and do se of Folltropin, for any of variables evaluated were observed (SAS Institute, Inc. Cary, NC). These results are product of the first year of evaluation and this project will continue for four more years.

GENETIC SELECTION FOR NATURAL DISEASE RESISTANCE AGAINST BRUCELLOSIS AND FOOT-AND-MOUTH DISEASE VIRUS IN COLOMBIAN CRIOLLO CATTLE

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OBJECTIVES

Phase I (2 years)

- To phenotype up to 250 BON cattle for resistance (or susceptibility) to virulent *Brucella abortus* using an *in vitre* peripheral blood monocyte-derived macrophage bacteristasis assay.
- To phenotype up to 250 BON cattle for resistance (or susceptibility) to virulent foot and mouth disease virus, serotype O, using a quantitative *in vitro* fibroblast or macrophage assay to assess the rate and magnitude of viral replication, and serum neutralization assay for protective antibodies.
- To establish a repository of frozen leukocyte pellets, fibroblasts and DNA from each BON sampled for molecular genetic analysis.
- To select two brucellosis and foot and mouth disease resistant and two susceptible BON sires, and twelve brucellosis and foot and mouth resistant and twelve susceptible BON dams as founders of the genetic study herd.

PHASE II (3 years)

- To establish a BON genetic study herd through *in vitre* fertilization and embryo transfer technology sibships of 30 each from RxR, RxS, SxR, and SxS muting from *in vitro* phenotyped founders.
- To *in vivo* phenotype 15 siblings from each muting by standardized challenge with virulent Brucella abortus and/or foot and mouth disease virus, serotype O.
- To produce and *in vitro* phenotype backcross progeny as a basis for genetic segregation and linkage analysis to identify molecular genetic markers of natural resistance against brucellosis and/or foot and mouth disease in criollo cattle.

SUMMARY

The capability of cattle to resist or tolerate bacterial, viral or parasite pathogens has a strong genetic base, therefore the objective of this project is to identify genes (or molecular markers) that control natural resistance against infectious diseases of economic importance.

To achieve this goal, it esa initially proposed to use functional *in vitro* tests to characterize by phynotipe the Colombian criollo cattle, Blanco orejinegro breed(BON) with regard to their specific natural resistance to brucellosis and foot and mouth disease.

Macrophage purification assays from BON cattle peripheral blood to be used in *Brucella abortus* infections have been performed. Several modifications from the original macrophage purification protocol were introduced b the research team at Universidad de Antioquia and then transferred to researchers at CORPOICA.

Based on the *in vitro* standardization of fibroblast cultures from cattle ear tissue, titration of foot and mouth disease virus type O was performed in 47 cultures from different BON cattle. Partial results suggest a lower permissiveness of fibroblasts to support foot and mouth disease virus replication with respect to that observed in BHK21 cells (control). Infectivity indexes ranged between 0.437 and 1.0. The distribution of these indexes are shown in the following table:

Infectivity index distribution of foot and mouth disease virus O1 Campos in fibroblast cultures from Blanco orejinegro (BON) cattle ear tissue.

Infectivity index interval °	No. of Cattle
0.4<0.5	1
0.5<0.6	1
0.6<0.7	7
0.7<0.8	8
0.8<0.9	13
0.9<1.0	7
S.I	10
Total	**47**

N.I.= Without information. Cells were not suitable for virus titration

a = Infectivity index TCID 50/rnl in fibroblasts / TCID50/rnl in BHK21 cells

Presently, there is a collection of leucocytes, fibroblasts, and DNA samples from about 120 BON cattle for further genetic marker analysis.

This information will be used to select and characterize phenotypically a breeding stock of bulls and cows through in vivo challenge with virulen pathogens to produce cattle lineages, mostly through embryo transfer of inbred embryos that segregate for these characteristics. These core families will be the source for an in-depth study to identify genes coding for natural disease resistance to brucellosis and foot and mouth disease. In addition, the identification of these markers will aid in the select ion of populations resistant to these pathogens, which will reduce the dependence on antibiotics and vaccines as an alternative for the prevention and control of these diseases.

MOLECULAR AND POPULATION GENETICS OF COLOMBIAN CRIOLLO CATTLE

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OBJECTIVES

- To obtain nuclear DNA from blood and establish fybroblast cell lines of the seven criollo cattle breeds.
- To collect data of mitochondrial DNA sequences and nuclear microsatellite information about population genetic data of the seven criollo cattle breeds (polymorphisms).
- To determine the phylogenetic relationship between the seven criollo cattle breeds and their relationship to other bovine breeds.
- To identify molecular markers (microsatellites) associated with economically relevant traits (production, reproduction, disease resistance, adaptation).

SUMMARY

Some Colombian Criollo Cattle breeds (Blanco Orejinegro, Casanare, Chino Santandereano, Costeño con Cuernos, Hartón del Valle, Romosinuano and San Martinero) have some special characteristics that have allowed them to adapt to local conditions. These characteristics include resistance to internal and external parasites, ability to tolerate extreme environmental conditions, such as high temperature and humidity, ability to strive on low quality forage, high reproductive efficiency and resistance to infectious diseases.

Every one of these breeds represents a unique natural resource. To make the best use of this resource, it is vital to understand the genetic potential and the phylogenetic history of these breeds. The purpose of this project is to use the mitochondrial genome sequence and nuclear microsatellite information to determine the phylogenetic relationship and the origin of these breeds and to map the economically relevant genetic traits of these unique breeds. With this information, we will analyze the molecular and population genetic bases of the Colombian Criollo Cattle. This knowledge is important to develop future breeding strategies and to make rational conservation efforts.

Partial results obtained to accomplish the first objective are presented on the table 1. It is observed at this time, a considerable amount of samples from each one of the seven Colombian Criollo breeds. Some of this material, has been sent from CORPOICA to Universidad de Antioquia and Texas A&M university.

Table 1. Samples of leukocytes, DNA and fybroblasts processed from animals of the seven Colombian Criollo Cattle breeds.

BREED	No. BOVINES	LEUKOCYTES (No. Vials)		DNA		FIBROBLASTS (Vials frozen)
			GOOD	REGULAR	DEGRADED	
Casanare	42	38(98)	36	3	3	7(38)
Costeño con cuernos	25	25(76)	25	2	-	11(24)
Romosinuano	25	25(70)	20	2	2	11(26)
BON	113	106(200)	73	-	23	18(57)
Hartón de Valle	22	19(89)	6	-	16	18(61)
Sanmartinero	47	34(76)	44	-	3	5(21)
Chino Santandereano	5	5(10)	2	3	-	-
TOTAL	279	252(619)	206	10	47	70(227)

Table No. 2 shows the results obtained from the evaluation of 11 microsatellites obtained from Texas A&M University.

Table 2. Partial results obtained from 11 microsatellits evaluated in the seven Colombian Criollo Cattle breeds.

Microsatellite	No. of alleles	No. of the marked breeds
BM4307	6	3
BM723	6	6
BM4311	2	2
BM6501	2	2
BM1905	1	7
HEL5	5	3
ETH225	3	6
ADCY2	0	0
INRA005	5	3
INRA063	3	7
ILST005	2	7
MAF45	0	0

The data showed on these tables are partial results from the first part of the project. The total project includes the evaluation of at least 30 microsatellites to have information from each chromosome of the bovine genome. This project has spent two of the five years proposed to accomplish the objectives.

MOLECULAR AND SEROLOGICAL CHARACTERIZATION OF *Leptospira hardjo* AND *Leptospira spp.* STRAINS ISOLATED FROM DAIRY CATTLE IN THE SAVANNA OF BOGOTA

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OBJECTIVES

- To attain knowledge concerning the distribution of *Leptospira* serovars, more specifically serovar hardjo, in dairy catlle in the savanna of Bogota, by developing and standardizing a methodology for the inter and intradifferentiation (Hardjoprajitno and Hardjobovis).
- To isolate, culture, serotype and gather information about the prevalence of strains of the different *Leptospira* serovars found in clinical-pathologic material from dairy catlle of the savanna of Bogota.
- To determine similarities and differences between Hardjoprajitno and Hardjobovis strains by total genomic DNA analysis using restriction enzymes.
- To distinguish between strains of serovar *hardjo* and between other serovars using serologic cross-reactions for which strain specific (Hardjoprajitno and Hardjobovis) and serovar-specific (*hardjo*, icterohaemorrhagiae, balcánica, javanica and vietnam) antigens will be used.

SUMMARY

Leptospirosis is a zoonotic disease first diagnosed many years ago in different animal species in Colombia. Internationally it is know as one of the most important bovine bacterial diseases from the reproductive point of view (gestation loses) and therefore from the production point of view (pregnancy interrumptions and decrease of milk production). Serological survey have been performed during the last twenty years in different catlle raising areas of Colombia showing an average prevalence of 21.7%, nevertheless there has not been research and divulgement enough to know the extent of the impact of the disease on the national herd. Studies that determine the differentiation and distribution on *hardjo* serovars strains (hardjobovis and hardjoprajitno) and other serovars strains present in the Colombian bovine are not know. Taxonomic differences among the members of the genus *Leptospira* imply at the same time differences in their pathogenicity, antigenicity and immunogenicity. Because of this, the understanding of these differences is indispensable in disease in animal populations (including man).

The purpose of the present project is to analyse and compare the total genomic DNA of the different serovars more specifically the strains of serovar *hardjo* (Hardjoprajitno and Hardjobovis), using molecular biology techniques, such as restriction endonuclease digestion, polymerase chain reaction (PCR) and/or Random Amplified Polymorphic DNA (RAPD); similarily, to distinguish serovars from leptospira

isolated from dairy cattle in the savanna of Bogota intra and interspecifically (*hardjo* icterohaemorrhagiae, balcanica, javanica and vietnam) using serologic cross-reactions with strain specific antigens. In this way we intend to shed light on the etiology of leptospirosis, learn about the distribution of serovars and determine their epidemiologic implications.

About the use of molecular biology techniques we developed the metodology to culture leptospiras in small-scale, isolate DNA, and standarize the restriction endonucleasa digestion. With restriction endonuclease analysis with did not find until know similarities to think that the strains under study are Hardjoprajitno or Hardjobovis, so the objectives of this project gain more importance with respect of the presence of the serovar *hardjo* on the bovine herd. We think that one topic that is very important is to improve the isolate metodology in order to increase the possibility of successful on strains isolation.

VALIDATION OF PCR FOR SPECIFIC DIAGNOSE OF BOVINE TUBERCULOSIS

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OBJECTIVE

- Comparison of specificity and sensitivity of PCR test and microbiological and clinical-epidemiological methods for diagnosing Mycobacterium bovis.
- Standardization of methods for chromosomal DNA isolation of Mycobacterium bovis in biologic samples for a widespread use of PCR.
- Development of a system for minimizing the possible problems of false positive and false negative results that occurs normally in PCR.
- Typification by biochemical reactions of different mycobacteria strains, isolated by culture in order to validate the specificity of PCR.
- Typify subspecies obtained from positive isolations of M. bovis by RFLP technique.

SUMMARY

The lack of reliable and quick diagnose methods for detecting bovine tuberculosis is one of the major causes of the failure of control and eradication campaigns against it. This research group already developed a diagnose method that proved to be 100% specific and highly sensitive. The developed method is the only capable of distinguishing between human and bovine tuberculosis in only one step. With this project, the groups aims to validate PCR and determinate its specificity and sensitivity, as well as its negative and positive predictive value, confronted to the traditional bacteriological diagnose.

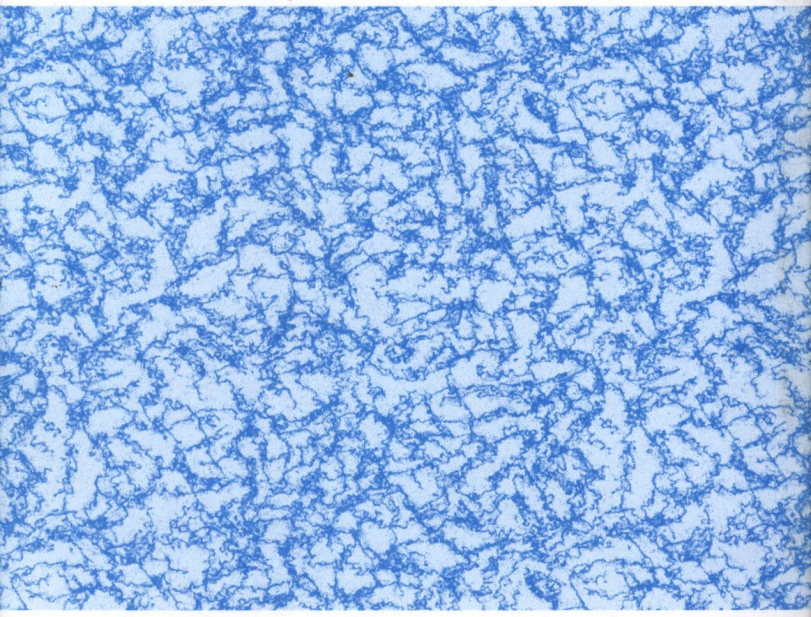
Isolation methods of M. bovis chromosomal DNA from biological samples for widespread use in PCR have been standardized from 4 different strains of the mycobacteria. The method is mechanical breaking by Bead Better, with silicon-zircon spheres (Biospect Products, Barlesville, USA) that shows a sensitivity similar to that of the enzymatic breaking and allows processing a bigger number of samples in a shorter time.

The results of microbiological processing and PCR were compared, and the result showed a relationship between the number of recovered bacteria and the intensity of band in PCR. PCR shows a unique amplification band corresponding to 500 pairs of basis.

The isolation of genomic DNA for RFLP study was achieved by CTAB method. The fragment used as probe was obtained from hydrolysis with Sma I of Bluescript vector.

The 765 pairs of basis fragment was used as probe. The isolated DNAs from M. bovis samples were hydrolyzed with PvuII enzyme, and then transferred and hydrided with the 765 pb fragment. In the RFLP/PGRS analysis, the four isolations showed polymorphism patterns of identical PGRS with approximately 17 bands, implying a genetic relationship between isolations.

65 milk samples and 60 blood samples have been analyzed by PCR and none of them has showed positive results, when analyzed in agarose gel. For this reason , molecular hybridization studies have been undertaken, using a marked probe (ECL Amersham). The dot blot system has identified 16 positive samples.



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