# ANNUAL REPORT 2003

## *PROJECT IP-06*



# Tropical Fruits, a Delicious Way to Improve Well-being

CIAT
Centro Internacional de Agricultura Tropical
International Center for Tropical Agriculture

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# Title: Tropical Fruits, A Delicious Way to Improve Well-being

## *1 Researchers:*

**Major contributions to this years work in the Tropical Fruits Program were made by the following persons:**

**Homologue Development**: Peter Jones & William Diaz in the development of Homologue algorithms and software; James Cock, Simon Cook, Rachel O'Brien, Peter Jones & Thomas Oberthur in the area of conceptialization development of new projects; Xavier Schelderman (IPGRI) and James Cock in the establishment of an inventory of neo tropical fruits. James Cock coordinated these activities.

**Participatory selection**: Juan JairoRuiz, Vanessa Segovia, Edith Tabares, Fernando Hincapié, James Cock, Luis Alfredo Hernández, Carlos Quiroz, F. Parra, and Zaida Lentini all made major contributions to this cross disciplinary effort on lulo. Corporación Biotech, Alvaro Mejia and James Cock were active in the work on propagation and selection of sour sop; Elizabeth Alvarez and C.A. Ospina carried out the extensive work on Antrhacnose.

**Thematic Tropical Fruits Network**: Liliana Rojas and Jenny Correa

**Fruit Flies in two areas of Colombia:** Tony Bellotti, Maria del Pilar Hernández and Monica Lucía Marín.

**New Research Policies for Fruit Crop Research in Colombia:** James Cock, Ximena Rueda (MADR), Juan Jaramillo (CORPOICA)

Many others made important contributions to discussions and provided support and services to the program. Their anonymous contribution is hereby acknolwledged. A special mention is reserved for Adriana Cardona the adminstative assistant of the program.

## *2 Cooperators:*

Within CIAT: Integrated Pest and Disease Management (PE-01); Land Use in America (PE-04); Rural Agroenterprises SN-1; Participatory Research SN-3; use of Genetic Resources SB-2.

Outside CIAT: National Programs: Colombia (CORPOICA, SENA, Corporación Colombia Internacional); IPGRI, Missouri Botanical Garden, University of Queensland (Australia); PROTA-University of Wageningen (Holland); ICRAF; GFAR; University of California Berkeley, University of Florida

# *3 Highlights in 2003*

The Centre's efforts in tropical fruit research and development in 2003 concentrated on (a) consolidating the basic strategies developed during the previous year and developing and implementing an operational plan and (b) setting up a more solid and stable funding base.

The program has suffered from extremely limited and uncertain funding during the year and future funding is extremely uncertain: this situation evidently makes coherent long term planning difficult. The assured resources of essentially one internationally recruited staff member (75%) with limited support are being used to (i) to search for financing (ii) to initiate research, based on the long term strategies, that will help attract funds and (iii) to continue with research to resolve problems identified by the Ministry of Agriculture and Rural Development of Colombia and (iv) to continue with the Sour Sop project funded by Spain (confirmed in the second semester of 2003).

In this report we present the funding situation, modifications to the overall strategies, and research and development activities.

## 3.1 Funding.

The program is at present financed principally by a small amount of core, part of the Colombian contribution and the Spanish Government. The present financial status is not sufficient to implement the strategic plan, and hence an increased funding base is required. The possible sources of future funding are an increase in the allocation of core resources as seed capital to set up the programme, special project funding to implement specific components of the strategic plan and contract research. At present the Centre has assigned less than 2% of its projected unrestricted core budget to the fruit Fruit Program and its share of administrative costs for 2004. This not only places the Fruit Program in a difficult situation due to the low level of core funds themselves, but it also severely restricts the programs capacity to obtain special project or contract research funds. Most CIAT special projects or contract research projects are heavily subsidized by core, normally in the form payment of a large proportion of the salaries and administrative costs directly from core. Those programs that have a small core assignment are not able to use core as leverage to subsidise special projects and make them lower cost and hence more attractive to donors. The fruit program lost the opportunity to carry out an interesting contract research project on Integrated Pest Control of Cape Gooseberry (*Physalis peruviana*), that was highly rated from a technical point of view, due to the high cost of the project: the project was eventually assigned to a university which placed very low charges for scientists time. The situation of the Fruit Program is further complicated by the fact that many donors are looking for projects which will have a direct impact on poverty in a short period of time: this is difficult to achieve in a new program that is starting from an extremely narrow knowledge base with few research results in the pipeline. A consequence of this situation is that the future directions of the program are likely to be more dependent on the whims of specific donors than on the lines laid out in the strategic plan.

The program is at a crucial point where it requires seed capital to build up a reputation so that it can present itself to donors as a viable unit that has an established research capacity. Once the program is functioning and has reached critical mass it should obtain special projects that build on the demonstrated knowledge and expertise developed within the program.

The financial dilemna of the Fruit Program is that without a reasonable assignation of seed capital to generate knowledge and expertise the program it will not be able to attract funding. The solutions to this conundrum are not clear at present. The possibilities, all of which are being actively sought, are:

1. To increase substantially the core budget assigned to the Fruits Program either directly or through collaboration with core financed units within CIAT
2. To attract a major donor interested in providing long term support for the development of an International Fruit Program
3. Package the components of the proposed Fruit Program into individual projects and search for financing of the individual packages.
4. Incorporporate components of the Fruit Program into project proposals jointly presented by the Fruit Program and other projects.

During the year a large number of concept notes and project proposals were prepared, often in collaboration with other entities within and outside of CIAT, for presentation to several potential donors under the strategies outlined above. These included: ***The development of Homologue and CropIdent to determine what to grow where; Better informed decisions through the integration of local and scientific knowledge; Selection and Propagation of Proven Perennial Fruit Trees; Bringing information symmetry in small holder coffee systems for intelligent production and transparent marketing of high value products; Rapid selection of Sour Sop (Annona muricata L.) suitable for specific agro-ecological conditions*** (presented to Colciencias by Corporaración Biotech with participation of CIAT); ***Integrated pest management for Cape Gooseberry (Physalis peruviana),*** presented with the National University, Bogotá, Colombia; ***Genetic resources of native and exotic fruit species in the Brazilian semi-arid region: improved livelihoods through consolidated agribusiness in the irrigated areas of the São Francisco basin*** presented to the Water Challenge Program by EMBRAPA; ***Zonas Aptas Para Producción De Frutas Y Hortalizas Especificas; What to Grow Where; Technical Assistance to Strengthen the Production of Alternative Agricultural Commodities in Colombia and other Andean Countries;*** and ***Flowers in the Park.*** Colciencias approved the project on rapid selection of Soursop, with the financial support destined to Corporación Biotec and CIAT providing counterpart support from the final year of the Sour Sop project financed by Spain. The project What to Grow Where was approved for funding, at a reduced level, by the USAID linkage grants scheme.

## 3.2 Strategies

The mission of the Tropical Fruits program continues to be: "to use science, technology and modern information technology to provide information and support to partners in the public and private sector that promote production, processing and marketing of tropical fruits by rural communities which leads to increased wealth and improved welfare for present and future generations in the countryside."

The strategy continues to be to provide support for the development of Tropical Fruits in general without concentrating on any one particular fruit species. At the same time we recognize that to

develop general principles we will need to carry out research on specific fruits. In general in these cases we try to ally ourselves with over agencies, such as the development of techniques for rapid selection of perennial species in which Sour Sop is the model species and we work closely with Corporación Biotech.

The strategic research areas have been reviewed in the light of the possible donor interest and the expertise that we have in house. It is difficult to develop coherent project proposals in fields in which we do not have in house expertise.

The program is emphasing the following areas:

- Targeting which crops or cultivars will grow well in particular conditions
- Generic research of general applicability to many fruit crops
- Agro-enterprise development
- Contract research

Market intelligence, which was considered last year as one of the strategic initiatives has been place on hold as we do not have a great deal of in house expertise in this area, and furthermore several other agencies are active in this particular field. In the area of generic research of general applicability to many fruit crops we have not been able to obtain financial support for the flowering and post harvest deterioriation initiatives. On the other hand there has been limited donor response in the area of developing technologies for rapid selection of improved genotypes of perennial fruits and we have moved ahead in this area.

## 3.3 What can be grown where

In last years annual report we noted that in agriculture one of the foremost questions is "What can be grown in a specific site". Furthermore the development of specialized software (CropIdent™ & Homologue™) was suggested as the first step in the development of a system to target what crops or cultivars will grow well in particular conditions (see section Homologue). CropIdent requires to be supported by databases on the individual plant species or cultivars, and work commenced on the establishment of a working inventory of Neo Tropical Fruits.

We are also progressing on the development of the user interface for the software. In addition arrangements have been made with the University of California, Berkeley (USAID linkage funding) to develop methodologies for combining local knowledge, scientific knowledge and expert opinion related to which crops succeed under which biophysical, socio-economic and management conditions. These methodologies will then be incorporated into software modules to provide appropriate, user-friendly tools for each situation along with farmer or farmer group accessible outputs.

If a particular site is georeferenced the climatic conditions of that site can be accurately estimated from existing climate databases. Soils on the other hand are spatially much more heterogeneous and methodologies for on site evaluation of soils are required for successful use of Homologue and CropIdent. Work has commenced on the development of a Rapid Soil Appraisal (RSA) methodology. Revision of previous work suggests that the RSA can be based on the work in Mexico of Siebe, Jahn and Stahr of the University of Hohenheim.

A first step in the setting up of CropIdent is to have a full inventory of all the potential crops. In the case of tropical fruits we have initiated the establishment of a readily accessible database of Neo Tropical Fruits. This work is based on the ethnobotanic inventory of neotropical fruits currently on the web which was developed by CIRAD-FLHOR and IPGRI, based on original work by Fouqué. This inventory was conceived as an electronic book, and its structure is not appropriate for eventual incorporation into CropIdent and linkage to Homologue.

In conjunction with IPGRI the information in the electronic book is being transferred to a structured database. During this process additional information is being added with emphasis on geographical distribution of the species, and the availability of germplasm. Linkages are being established with such organization as the Missouri Botanical Garden which have extensive databases on tropical plants with massive information on the geographical distribution of species.

## 3.4 Participative Selection of Perennials

In spite of the attractive nature of new varieties of fruit trees as a means to increase rural income, there are few examples of successful formal breeding efforts to improve perennial fruit crops. The lack of success in breeding fruit trees is related to few long-term efforts to improve fruit trees, particularly in tropical species, and the long generation interval and period of testing required in formal breeding programs. Furthermore, with so many different fruit crops it is most unlikely that traditional research organizations will dedicate the time and resources necessary to structure comprehensive breeding programs similar to those developed for short season annual crops or food staples. On the other hand, most fruit tree varieties favoured and planted by farmers throughout the world are the result of selections made by farmers themselves, or researchers, from chance variation found in naturally occurring seedlings. The key to successful use of these selections is first of all to nurture them and recognise their value. Second is asexual propagation, which ensures that all the plants are genetically identical, but which is also carried out in such a manner that systemic diseases are not propagated.

A major potential limitation of participative selection of perernnial species is the location specificity of the elite lines, and hence the difficulty of transferring the technology. We are developing methodologies to determine the similarity of sites in different locations (see above) and this will be used to determine in which areas the selected elite lines are likely to be successful.

We have taken two species, Lulo (*Solanum quitoense*) and Sour Sop (*Annona muricata*) and are using them as model species to develop methodologies for farmers and traders to select superior materials that are found spontaneously in the field, multiply them and plant them in areas where they will perform well (see sections on Lulo and Sour Sop for full details).

Colombia is the center of origin for sour sop, and the crop suffers various problems caused by phytopathogens that have co-evolved with it, in particular, anthracnose. This fungal disease, caused by *Colletotrichum* species, is spreading as the crop expands into areas that are environmentally favorable for the fungus. As a result, anthracnose is now a major constraint to soursop production in Colombia. To support our work on Sour sop the nature of the disease was studied (see section on Sour sop).

## 3.5 Thematic Tropical Fruits Network

This year, making use of REDECO's Internet experience to generate a flow and exchange of pertinent, updated information, the Tropical Fruits Program and REDECO plan to create a thematic network of users interested in the theme of tropical fruits. The network will facilitate identification of the demand, capture, and diffusion of specialized information, and will promote dialogue between users interested in belonging to the network. From May 2003, the theoretical framework of the Thematic Network on Tropical Fruits was developed, and a survey prepared aimed at the REDECO community (see Thematic Tropical Fruit Network).

## 3.6 Fruit flies

There are certain groups of pests that consist of a species complex that can attack and damage numerous fruit species. The fruit fly complex is an example of a pest that can damage numerous fruit species, and hence its study falls within our strategy of research on generic problems of fruits. See section on Fruit Flies for full details

## 3.7 New Research Policies for Fruit Crop Research

The Colombian Ministry of Agriculture and Rural Development (MADR) requested assistance from CIAT in designing research policies to support tropical fruit based enterprises. Working closely with the MADR we have developed guidelines for more effective use of research resources on Tropical Fruits. Although the policies are directly related to the Colombian situation, many of the recommendations are highly relevant to many other developing countries. The development of strong national or local research capacity is a *sine qua non* for success in promoting tropical fruits and strengthening local capacity is an integral part of the program strategies (see section on Research Policies).

# 4 *Full Reports*

## 4.1 Homologue development

The tropical fruit program is deploying two major software tools to assist in its goal of long-range transfer of tropical fruit germplasm. CropIdent will use a large database of information on crop characteristics to match fruit crops to potential environments. However this database does not exist at present and must be built up as new information becomes available. Homologue will provide a complementary function and assist in the production of the CropIdent database. The basic concept is that a farmer's field (target area) will have homologues somewhere in the tropics. Once these are known the local crops in these areas can be investigated with the view to introduction in the target area. In this process information will be gathered that will provide input to the CropIdent database. We also envisage Homologue being used to extrapolate from a small number of characteristic sites where fruit crops are known to do well. By combining Homologue probability estimates from these sites into a 'cloud' of estimates we hope to overcome the minimum accession set restrictions of FloraMap.

### *4.1.1 Materials and Methods*

#### *4.1.1.1 Climate probability mapping*

We compiled a set Eigenvectors and Eigenvalues from 25 FloraMap analyses. We chose to estimate the first five that account for over 96% of the variance in almost all cases. We mapped the probability surface from the components that explained 96% of the variance at levels over p> 0.3. We calculated the pixel coverage to get an estimate of the geographic area of adaptation. The variance of the accession points was also recorded. The adaptation area and variance defined the adaptation range.

The studies were extended to 136 FloraMap analyses for a wide range of species, and we attempted to fit regression models to the eigenvectors and encountered a number of problems. The first is that the sign of the eigenvector is ambiguous. This normally does not matter, but in this case we had to write a special program to determine the correct alignment by trying the fit with the sign in both directions.

After various attempts to improve the model fits, including clustering the species by climate type, sorting by the number of accessions and eliminating unusual eigenvectors, we decided that the task was beyond resolution. Even if a good fit could be achieved with the individual eigenvectors the complication of fitting all at once with restrictions to maintain orthogonality would have made the exercise too complicated.

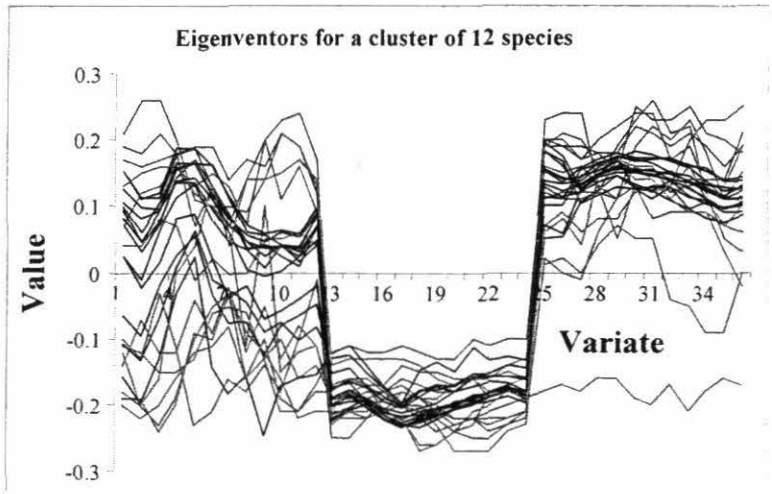



**Figure 1. Eigenvectors from 12 species in a cluster grouped by climate type**

**Figure 1**. Shows a typical example of a set of species clustered by climate type. The black lines are the fitted values from the best model fit, the red lines the actual eigenvectors. Although the R squared value is over 70% the fit is not good enough to serve.

We therefore decided to select a set of representative species and borrow the eigenvalues and eigenvectors as they stand. This had the advantage that, when they are applied to the actual observed data the probability model that results will be correctly orthogonal.

To do this a simple climate classification was devised: this was based on the temperature record dividing the observed climates into five groups by mean temperature and five by the variance of temperature. Table 1 shows the classification along with the colour index for the maps of the classification in figure 2. There are only 16 colours available in the palette for these maps so the colours cross classes in the table.

Climate Classes by Annual temperature characteristics

| Variance | Mean °C | | | | |
|---|---|---|---|---|---|
| | <15.5 | 15.5-19.0 | 19.0-22.0 | 22.0-24.0 | >24.0 |
| <1 | | | | | |
| 1-2 | | | | | |
| 2-5 | | | | | |
| 5-10 | | | | | |
| >10 | | | | | |

**Table 1**. Climate classification and color legend for the maps in Figure 2

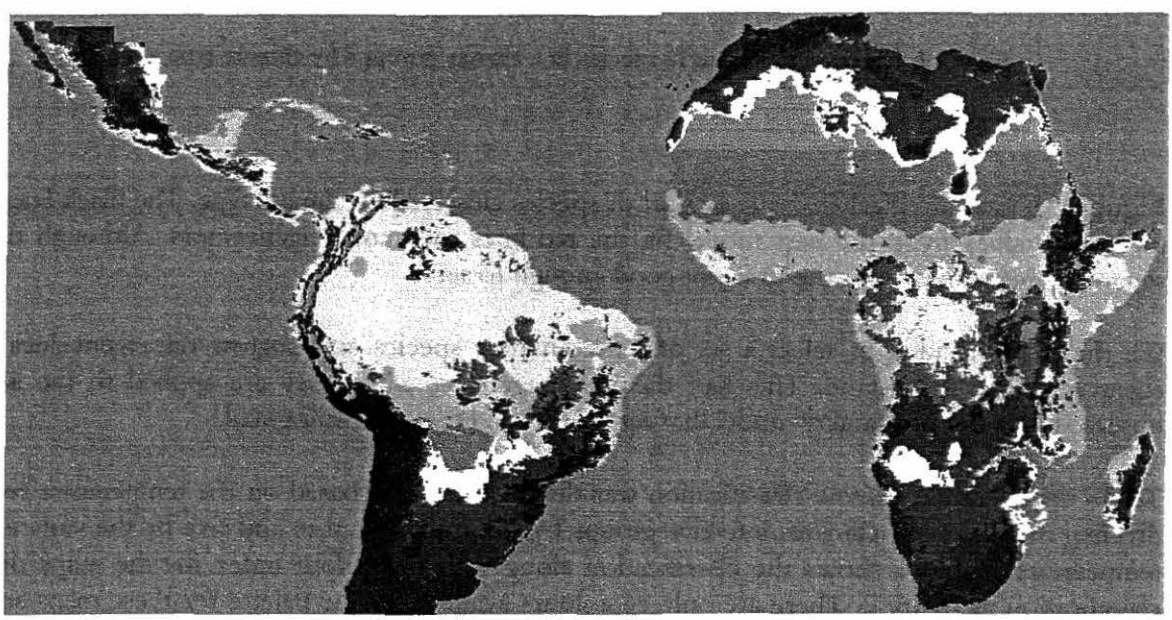


**Figure 2**. Climate classes for development of the generic climate probability model

The 136 FloraMap analyses were classified using this classification and representative species for each group selected:

| *Species* | | | | | |
|---|---|---|---|---|---|
| | Mean | | | | |
| Variance | <15.5 | 15.5-19 | 19-22 | 22-24 | >24 |
| <1 | *Oxalis tuberosa* | *Phaseolus leptostacheus* | *Phaseolus vulgaris* | *Vigna davyi* | *Vigna nervosa* |
| 1-2 | *Solanum nigrescens* | *Phaseolus leptostacheus* | *Phaseolus vulgaris* | *Vigna davyi* | *Vigna nervosa* |
| 2-5 | *Vigna parkeri* | *Vigna luteola* | *Vigna vexillata* | *Arachis burchartii* | *Arachis duranensis* |
| 5-10 | *Stylosanthes guianensis* | *Stylosanthes viscosa* | *Bemisia tabaci* | *Arachis glabrata* | *Arachis duranensis* |
| >10 | *Vigna ambacensis* | *Vigna venulosa* | *Manihot aesculifolia* | *Arachis cardenas* | *Arachis duranensis* |

Table 3. Representative species in each climate class used for the generic model.

Those in red are surrogates standing in where no species was yet available in that group. More data sets will be collected from FloraMap users over the near future and the table will be extended. The eigenvectors of the selected species were used to represent the model for each climate. The eigenvalues were easier to model and we derived regression models to estimate them for each climate. Based on this classification we wrote a generic routine for climate probability comparison. We derived new climate files and indices and determined that the compressed data can actually be stored in RAM in a space of about 20Mb. This makes the algorithm much more efficient. Subroutines were written to load the data, produce the climate probability shapefile and to return a climate record on request.

#### *4.1.1.2 Soil characteristics probability mapping*

This section of the work concentrated on working out how to provide and store the cumulative probability curves for the soil factors. The WISE soils database raw data provided enough individual profiles for many of the characteristics in a fair proportion of soils. From these we calculated variances for Depth, soil carbon, nitrogen, ph in water, KCl and $CaCl_2$, CEC, and texture as sand, silt and clay. There were a fair number of missing values in the resulting table, but most of these were filled with estimating regressions. This table of means and variances of the listed characteristics is now complete for all agricultural soils in the FAO classification.

The format for the soil characteristic integral takes 12 bytes. 2 bytes for the percentage of agricultural soil per pixel, 2 for the overall soil coverage per pixel and 8 single byte scaled increments. End points for the x-axis of each soil integral are held in a table separate from the pixel integral file. We have developed an interpolator function for this integral that can handle any shape of monotonic probability integral.

After a first read through the Latin American files we determined that the data for soil carbon nitrogen and CEC need transformation to normalize the distributions. Carbon needs a natural log transformation and both nitrogen and CEC need square root transformations. Other characteristics seem to be sufficiently normal to work without transformation. An encoder was

developed that takes all soil mapping units falling within a climate pixel, sorts out the agricultural soils and using the means and variances for each soil in each mapping unit compiles a probability integral for each soil characteristic. Figure 3 show the probability integrals for soil carbon for three random pixels in Latin America.

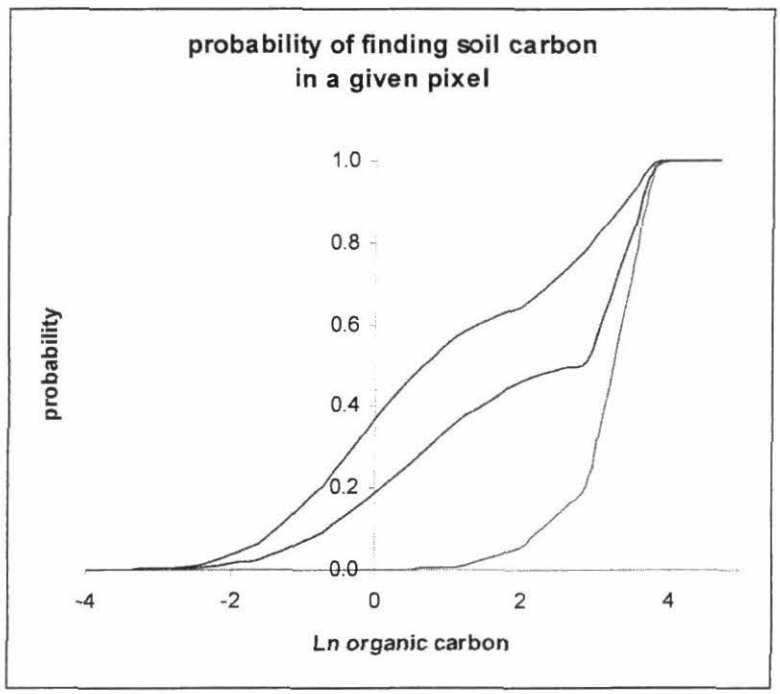



Figure 3, Selected pixels, the probability integrals for soil carbon.

### *4.1.2 User interface development.*

We have started development of the user interface. The point selection and mapping routines are being derived from those of MarkSim with the addition of capacity for displaying probability maps. The interface to the mapping dll is determined and passing structures defined. We have determined an efficient way of holding the large data arrays for access by the Fortran dll.

### *4.1.3 Results and Discussion.*

The object of this exercise is to produce a demonstration version of Homologue within this year for demonstration to donors for support for a full system. To this end we have restricted ourselves to using the existing 18km climate grids and some necessary function will not be developed until later. One of these is the allowance for correlation among the soil characteristics. This can be overcome but needs some theoretical work. Extending the product to more precise climate grids will have to wait until the new indexing systems are fully developed. Implementing these will require substantial change to the way the data is accessed in future versions but this is not envisaged to be a great problem.

## *4.2 Participatory Selection*

### *4.2.1* Selection of elite clones by famers using in vitro propagated plants of *Solanum quitoense* (lulo)

A large number of fruits of Andean origin have great potential to become premium products for local and export markets with a high economic return for the farmers. *Solanum quitoense*, locally known as lulo in Colombia and as naranjilla in other countries, is among these fruits. This species is native from Colombia and Ecuador, and it is normally cultivated between 700 and 2000 meters above sea level. Some of the main attributes of this fruit include its high level of vitamin C, and the sub-shrubby perennial growth amenable for cultivation in hillsides and inter-cropping, aiding soil conservation practices. Recently in Colombia, naranjilla changed from being a fruit of local fresh consumption to become an important industrial fruit for juice and yogurt products, increasing its market value. A major constraint for the rapid adoption of naranjilla by the local farmers is the limited availability of elite germplasm free of pathogens from clonal propagation. The high level of heterozygosity of this species is reflected in the high segregation of traits by its multiplication through botanical seeds. Rapid multiplication of high quality planting materials is of paramount importance. One of the main objectives of this project is to develop a protocol for *in vitro* propagation of naranjilla with application for conservation and rapid multiplication of elite clones free of pathogens. This protocol would facilitate the conservation and multiplication of high quality planting material aiding control of diseases which are one of the major constraints of this crop. In the previous two years we reported the development of an efficient protocol for the maintenance and propagation in *vitro* of clones selected from farmers field, for the plant regeneration from tissue cultures, and the preliminary evaluation of those plants in the field. This year we report the agronomic performance of those materials in the field, including quality traits of the fruit, as well the progress made to evaluate jointly with farmers the application of this technique at larger scale.

#### *4.2.1.1 Materials and Methods*

**Plant material.** High quality and elite clones provided by the Andean Fruit Center (Centro Frutícola Andino – CEFA) were used. This collection includes naranjilla with or without thorns commonly grown by farmers. The plants were propagated in *vitro* or plants were regenerated following procedures as reported by Segovia et al. in SB2 Annual Report 2002.

**Plant and fruit evaluations.** A small-scale field trial was conducted at 1700 m over sea level and a mean temperature of 22C to compare the growth, development and agronomic performance of regenerated plants respect to *in vitro* propagated clones. Fruits produced during a period of five months were harvested, counted, weighted, and classified by size according to standard scale. Premium size: > 5.5.cm diameter. Commercial size: from 3.5 cm to 5.5 cm in diameter. Not commercial: < 3.5 cm in diameter. The fruit maturation process (days from harvest to ripe to rotten) was evaluated using the Munsell color scale. Fruit quality trait analyses were conducted at the Valle University (Cali). These analyses included Brix grade at 20C, relative humidity, acidity percentage, vitamin C, sugar content, pH. A sensorial panel analysis was conducted to evaluate the fruits for its appearance, color, aroma and flavor as fresh fruit or processed in juice, and compared with those available in the supermarket.

**In vitro introduction of plants from greenhouse or field.** Greenhouse or field grown plants were tested to select the best protocol for the *in vitro* introduction of elite farmer's clones from the field. In order to establish plants in the greenhouse, the recovery of plants from axillary buds using shoot stakes was tested. Stakes of 20 cm in length from adventitious shoots with 2-3 axillary buds were taken from selected plants in the field and disinfected first with 3 ml/l of fungicide propamocarb HCL *(Pervicur)* for 5 min. The basal section of the stake containing the lower two buds was either: a) Cultured in water until root formation; b) Soaked in a solution of 10 mg/l NAA for 3 days and then transferred to water until root formation; or c) Potted directly in sterilized mix of soil, sand and sugar cane plant residues (Franco et al., 2002). Treatments (a) and (b) were aerated with a fish tank pump system. In order to establish plants *in vitro*, apical or axillary meristems from plants grown in the greenhouse or field were tested. Explants were surface sterilized and cultured *in vitro* on medium A instead of medium 17N (Segovia et al., 2002. SB2 Annual Report). Riphampicine (antibiotic) 100 mg/l was added to the medium to control bacterial infection from field explants, since the presence of trichomes in the tissues prevented thoroughly disinfections.

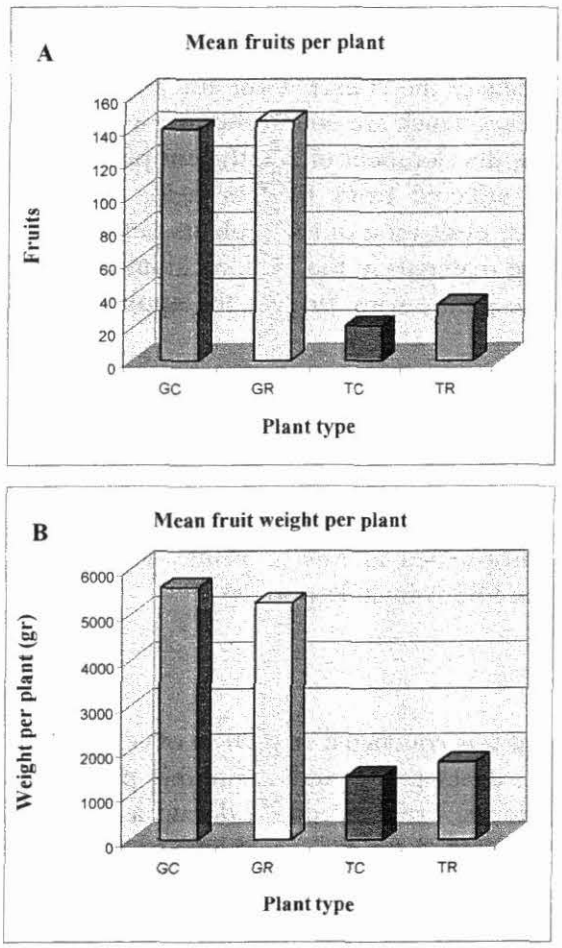



Figure 1. Fruit production in the field of *in vitro* propagated (C) and regenerated (R) plants derived from materials with thorns (T) or without thorns (G)

**Evaluation of the technology with farmers.** A total of 20 farmers from two regions of Colombia (Cauca y Huila) with commercial production of lulo were selected. Farmers were chosen based on the years of experience cultivating lulo, the number of lulo plants currently grown and their interest to participate in this initiative. A strategy was planned jointly with the farmers to evaluate the potential commercial use of *in vitro* generated plants as an alternative of planting material clean of pathogens. This activity was conducted in collaboration with IPRA, the tropical fruit project of CIAT, and Corpoica-Popayán.

*4.2.1.2 Results and Discussion*

Last year we reported no differences in plant growth and development between regenerated and *in* vitro propagated plants. Likewise, no significant differences were noted between the regenerated and *in vitro* propagated plants respect to the total number and total weight of fruits produced (Figure 1A). However, the clones with thorns (T) showed higher disease and pest susceptibility that significantly reduced fruit production

(Figure 1A). The average productivity in 5 months of harvest indicated a mean number of fruits per plant of 142 for materials without thorns (G) and 28 fruits for T (Figure 1A), and a total weight of fruits per plant of 5.58 kg for G and 1,57 kg for T (Figure 1B). About 80% of the fruits harvested from these clones showed a commercial size (from 3.5 cm to 5.5 cm in diameter). The rest of the fruits were classified as 15% small fruits (< 3.5 cm in diameter) for the G plants, and 10% premium size (> 5.5 cm in diameter) for the T materials (Figure 2). Commercial production of lulo may yield per year about 135 fruits/plant and 9 kg fruits/plant when cultured at a density of 3,000-plants/ hectare (3 $m^2$ per plant, CCI). In this experiment plants were also planted at a distance of about 3 $m^2$ per plant. Of the plants evaluated four were selected for their high yield potential. These plants produced 9 kg fruits/plant during the ½ year experimental harvest time. These plants were selected; seeds and stakes of these plants were collected, and grown in the greenhouse and *in vitro*.

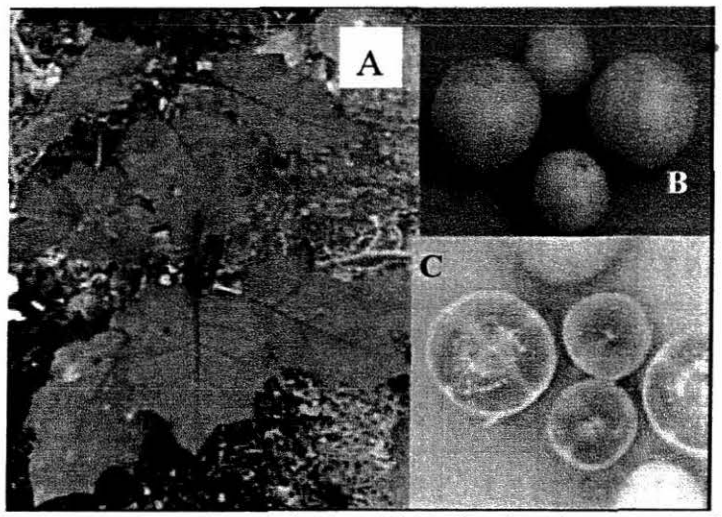


Figure 2. (A) Plant without thorns. (B) Fruits showing commercial and non-commercial sizes. (C) Dark green flesh that showed high acceptance by panelist in the quality trait assay

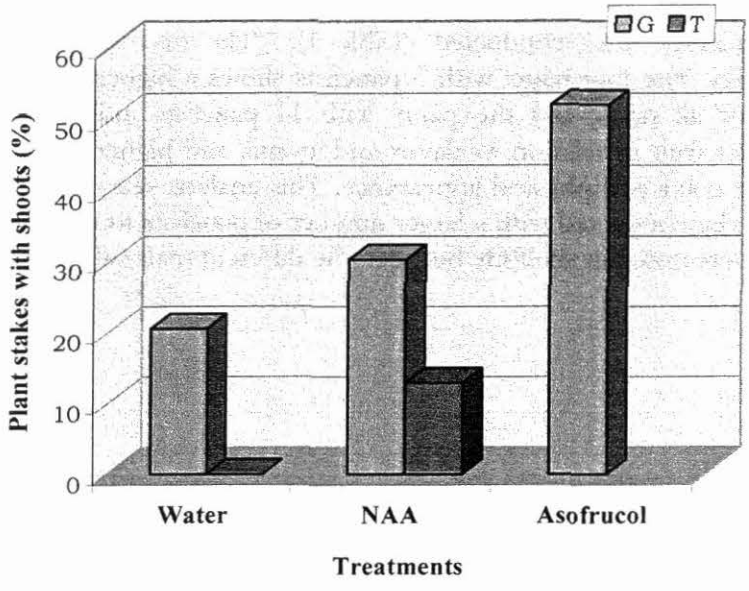
In relation to fruit quality, no significant differences were noted between the *in vitro* propagated clones, the regenerated plants and the commercial fruits bought in the supermarket for most chemical traits evaluated. The exception was the content of reducing sugar, which was lower in the supermarket fruit possibly indicating a longer post-harvest time respect to the experimental fruits at the moment the analyses were conducted (Table 1). The two sensorial analyses conducted gave different results. The first panel with 5 panelists shows a higher acceptance for the experimental materials for all traits, but the panel with 11 panelists indicated a higher acceptance for the supermarket fruit in relation to flavor and aroma, and higher acceptance for the experimental materials for color and physical appearance. This analysis seemed to be highly subjective, thus it may need to be conducted with a larger number of panelists to elucidate clearer preferences. No differences were noted in shelf life between the different materials.

Table 1. Chemical quality trait analysis of fruits from regenerated plants without thorns (GR), *in vitro* propagated plants without thorns (GC), regenerated plants with thorns (TR), *in vitro* propagated with thorns (TC), and bought in supermarket

| Trait | GR | GC | TR | TC | Super-market |
|---|---|---|---|---|---|
| Brix grade (at 20 °C) | 11.5 | 11.6 | 11.0 | 10.0 | 10.5 |
| Acidity (%) | 2.2 | 2.2 | 2.7 | 2.3 | 2.9 |
| Humidity (%) | 86.8 | 85.2 | 79.8 | 87 | 82.4 |
| Reduced sugar (%) | 4.6 | 5.5 | 5.0 | 4.9 | 0.7 |
| Total sugar (%) | 3.1 | 3.4 | 2.7 | 3.1 | 2.7 |
| pH | 3.2 | 3.2 | 3.2 | 3.3 | 3.2 |
| Vitamin C (mg/100g) | 38.2 | 37.3 | 48.8 | 64.2 | 39.1 |

Attempts to establish plants in the greenhouse from field grown materials indicated that the highest percentage of stakes with new shoots was obtained when the stakes were planted directly into soil in the greenhouse. About 50% of the stakes had healthy looking plantlets 1 month after planting (Figure 3). Explants derived from clones with thorns responded poorly, perhaps due to the weak stage of the donor materials since they were highly affected in the field. Once plants were established in the greenhouse stakes from greenhouse-grown plants produced profuse roots when treated with aerated water, which ease the mass clonal propagation of plants in the greenhouse. A high percentage of contamination was obtained when meristems were introduced *in vitro* directly from field grown plants. Explant survival (elongated shoots with roots) was increased from 40% to 60% when the antibiotic was added to the culture medium. No contamination was noted and at least 80% explant survival was obtained when meristems derived from greenhouse grown materials. Based on these results, successful *in vitro* introductions of elite materials selected in farmers fields can be achieved by establishing first clonally propagated plants in the greenhouse, and use these plants as donors for the *in vitro* culture of meristems. In the case a direct introduction from the field into *in vitro* conditions is needed, a more efficient surface sterilization protocol needs to be revised.

Figure 3. Percentage of stakes with new shoots derived from field-grown plants and treated in the greenhouse. (G) No thorns, glabrous. (T) Thorns.




A process was initiated to

test with farmers the suitability of using *in vitro* propagated plants as planting materials. The potential advantage on the in *vitro* source is the supply of pathogen free homogenous plants maintaining the selected traits of the elite materials. Farmers were selected from two sites with commercial production of lulo (Pescador, Cauca, and Tierradentro Cauca-Huila). These sites include small, medium, and large-size production farms. The farmers have between 2 years to 9 year of experience cropping lulo, and 200-5,200 plant-size farms. Farmers attended a workshop at CIAT with the objective to define jointly a strategy to evaluate the potential commercial use of the *in vitro* plants. The advantages and limitations of using *in vitro* grown plants were discussed. The farmers evaluated the plants at the CIAT lulo experimental plot. The criteria were established for the collaboration. Farmers are highly enthusiastic with the project, and a teamwork approach will be implemented. For each site, two nursery plots will be established. Farmers will select the best two plants currently grown in their fields based on productivity, fruit quality, and disease/ pest resistance. These plants will clonally propagated in the field, and explants will be established in the greenhouse at CIAT. Meristems from these plants will be introduced *in vitro*. Pathogen-free *in vitro* propagated plants will be grown in the nursery plots jointly with the field-clonally propagated plants, and seedlings derived from the seeds of the selected plants (standard propagation mode used by the farmers). The *in vitro*, clonal-propagated and seed-propagated plants will be compared throughout the production season. The nursery plots could also be used for the evaluation of new germplasm, one of the main needs identify by the farmers, in addition to assistance for an integrated crop and disease/pest management approaches. Corpoica plays a key role in these aspects, as CIAT is trying to strengthen their linkage to the project. Farmers selected their best elite materials and currently the plants are being clonally propagated in the field. Plants will be brought to CIAT to initiate their production *in vitro*. Field evaluations will be conducted first semester of 2004.

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### 4.2.2 *Genetic variability of the Colombian collection of soursop (***Annona muricata** ***L.) and related Annonaceae species***

Colombia is rich in species of the family Annonaceae (Murillo 2001). Many species which produce edible fruits belong to this family, mainly in the genera *Annona* and *Rollinia*, several of which are of actual or potential economic importance. These include: soursop (*Annona muricata* L.), cherimoya (*A. cherimola*), anon (*A. squamosa*), anon amazonico (*Rollinia edulis*), anona colorada (*A. reticulata*) and atemoya (*A. squamosa* x *A. cherimola)* among others.

The rich genetic variability in the country contrasts with the limited availability of well characterized cultivars. Commercial nurseries offer two or three cultivars of soursop and few of other species.

We developed a methodology to propagate selected trees of soursop through in vitro micrografting. This methodology can be used for the production of clean planting material of soursop and other related species. This technique is only commercially useful if

highly productive clones, of good agronomic performance, which produce fruits of good quality are identified.

The Colombian Corporation for Agricultural Research - Corpoica Palmira station maintains a collection of germplasm of soursop and related species, with 32 entries of soursop and 12 entries of other Annonaceae.

This activity is part of a project aimed to characterize, agro-morphologically and molecularly, using AFLPs, this collection and other available accessions (Royero *et al.* 2002, Mejia *et al.* 2002 and Saavedra *et al.* in preparation).

During 2003 we continued with the molecular characterization of the collection and with the analysis of the information produced.

**Methodology:**

Seventy eight accessions belonging to species of the genera *Annona* and *Rollinia* were characterized through the use of molecular markers of the AFLP type. The accessions were composed of 37 entries from *A. muricata* and 41 of related species, mainly from national germplasm banks of soursop and related species, maintained at Corpoica – Palmira, but also from banks of other institutions, farms, markets and commercial plantations (see annual report of 2002 for the list of accessions).

We applied the method of Dellaporta et al. (1983) for DNA extraction, using PVP 1 g $l^{-1}$ in the extraction buffer, and a chloroform / isoamylalcohol (24/1) cleaning step before DNA precipitation. The AFLP protocol followed was that described by Vos et al. (1995), using AFLP Analysis System I Kit (Gibco BRL). In preliminary trials we evaluated 15 different combinations of selective nucleotides for AFLP amplification. Then, we selected three combinations that displayed the highest and "most readable" polymorphisms between soursop species and/or

between Annonaceae species. They were **E-ACT, M-CAA (combination F); E-AGC, M-CTC (combination M); E-AGC, M-CAA (combination N).** Each accession was analyzed using two primer pairs. The combination of primers F and M was used for the soursop accessions and combinations M and N for the *Annonaceae* accessions.

Selective amplifications were size-fractionated on 6% or 4% polyacrylamide denaturing gels and visualized through silver staining. AFLP fingerprinting of each accession were converted into a similarity matrix, based on Nei and Li index (1979). The similarity matrix was analyzed using NTSYS (Rohlf, 1994) computer program. Dendrograms were constructed by UPGMA method (Sneath and Sokal, 1973).

**Results and Discussion:**

*4.2.2.1 Genetic variability of the different accessions of* Annonaceae

An example of the AFLP banding pattern produced by the different accessions is shown in Figure 1.

The comparison of the similarity matrixes obtained with the two different primer pairs, showed a high correlation (r=0.93-0.95 ) in the soursop accessions, as well as in the other *Annonaceae* accessions, indicating that both primer combinations detected a similar level of genetic variability, and that the data produced were reliable.

The dendrogram produced from the AFLP data of the *Annonaceae* accessions is shown in Figure 2. There are two main clusters, with similarity level of 0.10, the first composed by a single accession, Cadmia (*Cananga odorata*), an *Annonaceae* of Asiatic origin, included in this analysis as an out-group, and the second group includes the rest of accessions, which belong to the neotropical genera *Annona* and *Rollinia*. At a level of similarity of 0.55 (gray line in the Figure 2), nine subclusters can be detected (marked as groups G1 to G9 in the Figure 2).

Since the taxonomic classification of several accessions of these subclusters is known, it can be deduced that these subclusters represent at least nine different species, as follows: G1. *A. glabra*; G2. unidentified *Annona* spp.; G3. *Rollinia* spp; G4. *A. montana*; G5. *A. muricata*; G6. *A. purpurea*; G7. *A. reticulata*; G8. *A. cherimola*; and G9. *A. squamosa*. The *A. squamosa* x *A. cherimola* hybrids known as atemoyas, all fell within groups 8 and 9, which confirms their hybrid nature.

A low similarity, or in other words, a high variability was found in the accessions of the subclusters G3, G4, G7, G8 and G9.

Due to the low similarity presented by some accessions (0.75 or less) of the same subcluster, it is not clear if some of the subclusters such as G3, G4 and G7, are composed of accessions of one or more species.

Although some of the subclusters are represented only by few accessions, the variability found among them, can be exploited in breeding activities.

**Figura 1** Example of the AFLPs banding patterns of *Annonaceae* accessions obtained with the primer combination M (only the superior half of the gel is presented).

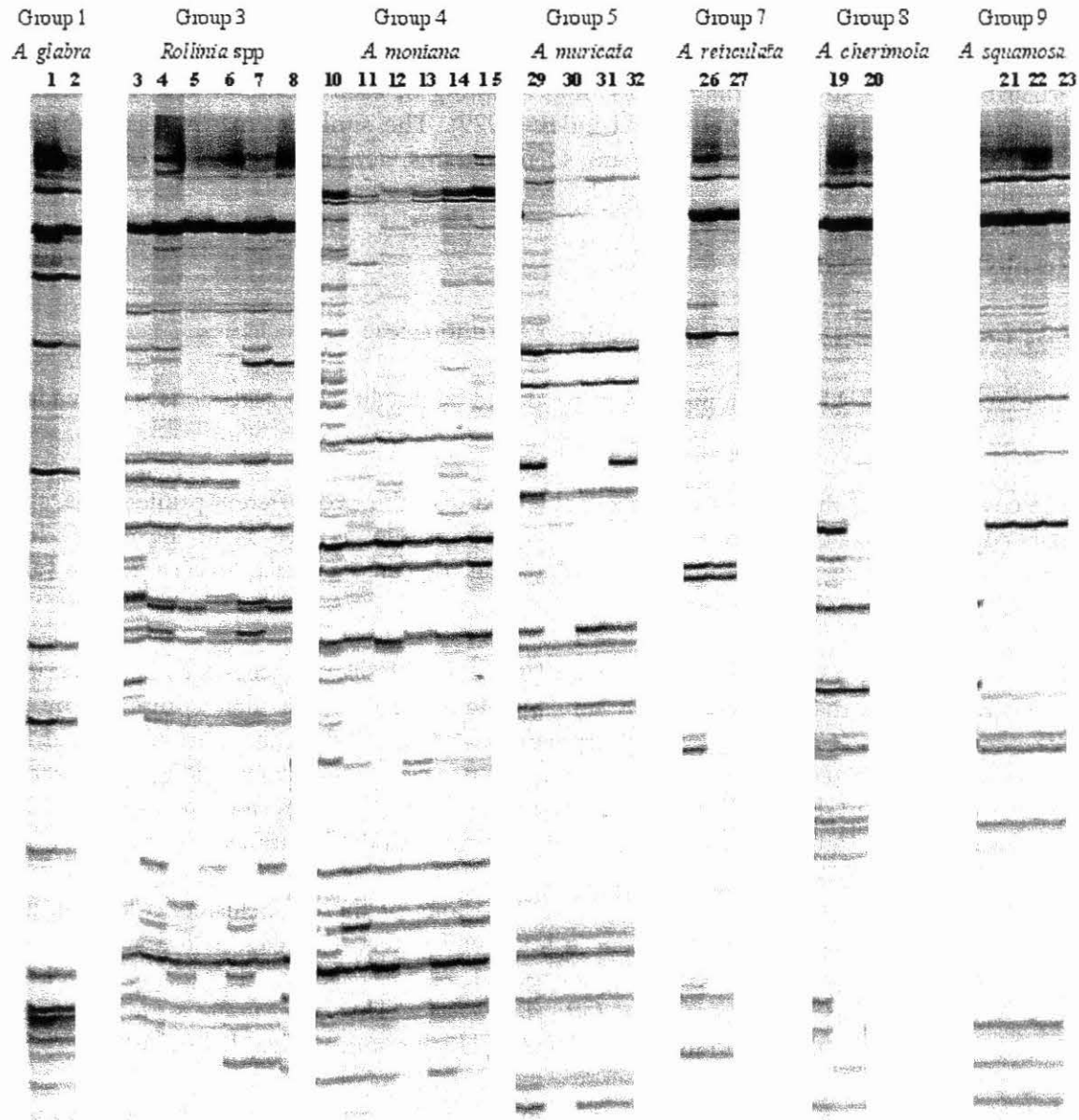
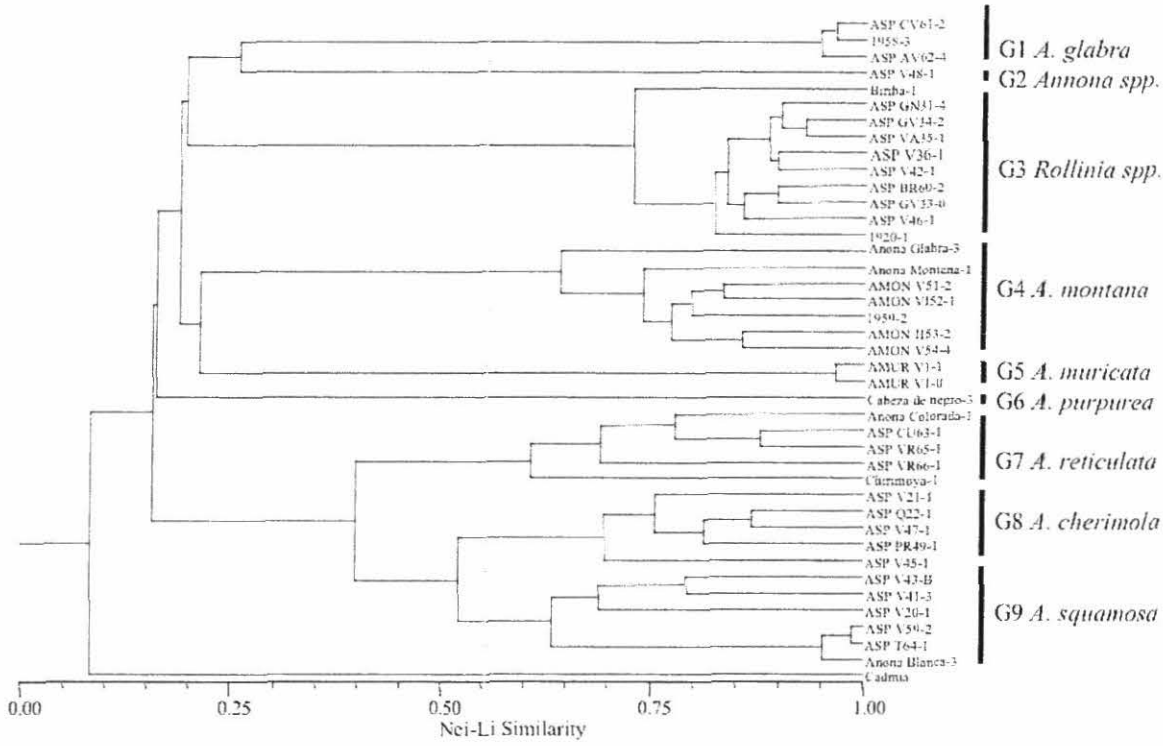


Figure 2 Genetic similarity tree of 40 *Annonaceae* accessions based on AFLP fingerprinting with the primer combinations M and N. On the right side of the dendrogram, the different subclusters found at a level of symilarity of 0.55 (grey line, subclusters marked with G1 – G9), and the possible species that each of them represent.




**Analysis of the genetic variability of the soursop accessions**

The 37 accessions of soursop presented similarities between 0.81 and 0.95. This similarity is higher than that observed in the subclusters of other Annonaceae 3, 4, 7 and 8 (corresponding to *Rollinia* spp., *A. montana*, *A. reticulata*, *A. cherimola* and *A. squamosa* respectively). No 100% similarity was found among the different soursop accessions analyzed, although they included two selected clones and seedlings derived from them (AMUR-H3-1 and AMUR-H2-1, derived from AMUR-H3 and AMUR-H2 respectively). This indicates that no duplicates are found among the different accessions.

At a level of similarity of 0.82 two subclusters can be observed. The subcluster 1 includes two accessions, the 6-3 of Palmira-Valle and the 2039-2 of Sonso-Valle. The subcluster 2 includes a total of 35 accessions of Venezuela (2014-2), Costa Rica (AMUR-H3-1), the Colombian departments of Magdalena (AMUR-M5-2), Antioquia (AMUR-A7-2), Cundinamarca (2015-1), Caldas (1918-5), Quindio (1946-10), and Valle del Cauca (the rest of accessions). There was no obvious relation between the origin of the accessions and the level of similarity presented by them. One explanation of this results can be that the accessions are not originally from the sites where they were collected, rather seeds have been transported by man over the centuries.

The accessions of soursop showed very low similarity (0.22) with the next most similar subcluster G4 (*A. montana*). With other subclusters the similarity was 0.19 (G1 and G3) or less (0.16; G6, G7, G8 and G9). This may explain why interspecific crosses of soursop with *A. squamosa, A. glabra, A. montana, Rollinia mucosa,* or *A. reticulata* have not yielded viable and fertile hybrids (Nakasone and Paull, 1998; Samuel *et al.* 1991; Mohd-Kahlid, 2002).

This suggest that the efforts in breeding of this species should be concentrated in the germplasm available within the species. Other species not included in this study, may be genetically closer to soursop, and these could possibly be used in soursop breeding.

The available variability of soursop at the interspecies level in the germplasm bank can be exploited to produce sexual hybrids from whose progeny, individuals with superior agronomic performance or fruit quality, can be selected.

The results of the present study stress the need to collects species with low representation in the germplasm bank, as well as of other species not yet represented in the collection.

### *4.2.2.2 Proposed taxonomic classification of the different accessions analyzed in this study*

The taxonomy of most of the accessions included in this study is clear and doesn't leave room for misclassifications for most of the subgroups. However, according to our results, a few accessions were incorrectly classified in the bank, (highlighted in table 1). In Table 1, we present a proposed classification of the accessions analyzed in this study according to AFLP similarity data. For some of the accessions, this classification has to be confirmed with other classical taxonomic techniques (underlined in Table 1).

Figure 3. Genetic similarity between *A. muricata* accesions, based on AFLP fingerprinting with the primer combinations F and M. The accession ASP-GV34 was used as an outgroup in the analysis. Accessions 1920-1 (*Rollinia spp.)*, 1959-2 (*A. montana*), and 1958-3 (*A. glabra*) were previously characterized as soursops. However, this tree does not group them with soursops.

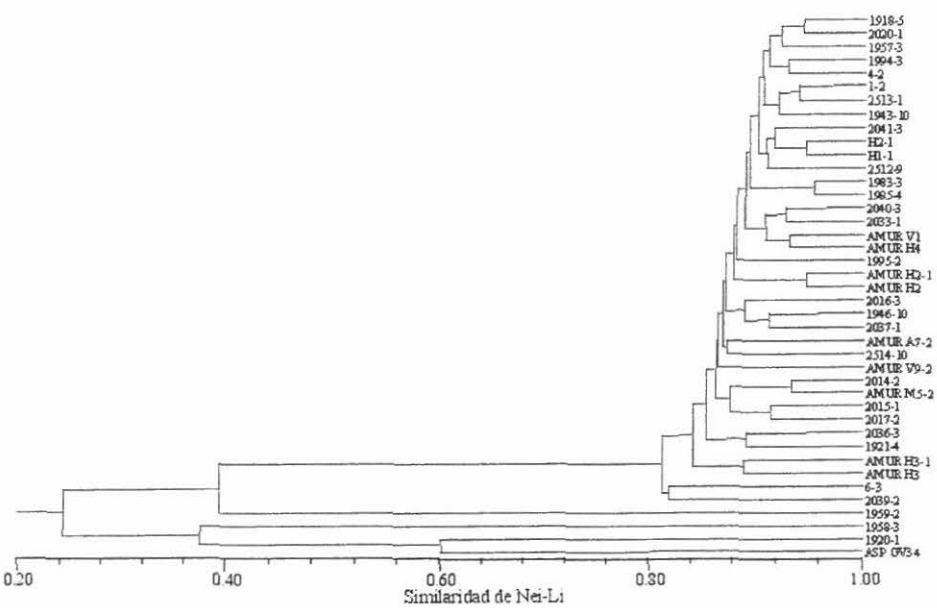


**Table 1. Proposed taxonomic classification of the accessions of *Annonaceae* characterised molecularly through AFLPs, and its distribution in Colombia (biogeographic zones).**

| SPECIES | COMMON NAME (spanish or portuguese name) | DITRIBU-TION IN COLOMBIA[1] | ALTITUDE[2] | REFERENCE[3] COLLECTION | CHROMOSOME # AND PLOIDY (Reference) | AVAILABLE ACCESSIONS |
|---|---|---|---|---|---|---|
| *A. glabra* | Pond apple, alligator apple (Anón liso, guanabanilla, guanábana de pozo) | car; pac | 0 - 200 | R. Romero 10506 [COL] | 2n = 4x = 28 NW Simmonds (1998) | ASP-CV61, ASP-A62, 1958 |
| *Annona muricata* | Soursop (Guanábana, catuche, graviola, zapote agrio, sorsaka) | and; amz; ori; pac; pu; snt; vc | 100 - 2000 | J. Cuatrecasas 9695 [COL][2] | 2n = 2x = 14 NW Simmonds (1998) | AMUR-V1, AMUR-H2, AMUR-H3, AMUR-H4, AMUR-M5, AMUR-A7, AMUR-V9, 1, 1918, 1919, 1921, 1943, 1946, 1957, 1983, 1985, 1994, 1995, 2014, 2015, 2016, 2017, 2020, 2033, 2036, 2037, 2039, 2040, 2041, 2042, 2045, 2511, 2512, 2513, 2514, 4, 6, H-1, H-2 |
| *A. cherimola* | Custard apple, cherimola, (Chirimoya, chirimorrinón) | and | 1600 - 1900 | S. Díaz 3162 [COL] | 2n = 2x = 14 NW Simmonds (1998) | ASP-V21, ASP-Q22, ASP-V47, ASP-PR49 |
| *A. montana* | (Guanábana cimarrona, guanábana del Chocó) | pac | 60 - 120 | H. León 332 [COL] | 2n = 2x = 14 Samuel *et al.* (1991) | AMON-V51, AMON-Vi52, AMON-H53, AMON-V54, 1959, Annona montana, accession classified as *Annona glabra* |
| *A. squamosa* | Sweetsop, sugar apple, custard apple (Anón, ata, anona blanca) | and | 340 - 1300 | J. Duque 3571 [COL] | 2n = 2x = 14 NW Simmonds (1998) | ASP-T64, ASP-V59, anona blanca |
| *A. squamosa* x *A. cherimola* (hybrid) | Atemoya | Introduced from Florida, USA | Good adaptation to the Cauca Valley 1000 | | 2n = 2x = 14 NW Simmonds (1998) | ASP-V20, ASP-V41, ASP-V43, ASP-V45 |
| *A. reticulata* | Bullock's heart (Anona colorada, mamón, corazón de buey) | and; car | 500 - 1900 | J. Walker 259 [COL] | 2n = 2x = 14 Nakasone y Paull (1998) | ASP-VR65, ASP-VR66, Anona Colorada, ASP-CU63, Chirimoya |
| *A. purpurea* | (Soncoya, guanábana tosete, cabeza de negro, manirote) | car | 300 | H. Cuadros 4635 [COL] | | Cabeza de Negro |
| *Rollinia spp.* possibly *all R. mucosa* | (Anón amazónico, biriba, condesa, corosal) | and; amz; pac | 20 - 1000 | G. Lozano 420 [COL] | 2n = 6x = 42 Samuel *et al.* (1991) | ASP-GN31, ASP-VA32, ASP-GV33, ASP-GV34, ASP-VA35, ASP-V36, ASP-BR60, ASP-V46, 1920, ASP-V42, Biriba |

[1]Murillo-A, 2001; The distribution correspond to biogeographic zones of Colombia: Pacific (pac), Amazon (amz), Caribbean (car), Orinoco (ori), Andean (and) regions; [2]Meters over sea level; [3][COL] Herbario Nacional Colombiano.

**Conclusions:**

The similarity analysis with AFLP molecular markers provided an estimate of the genetic variability of the *Annonaceae* accessions.

Each accession showed a different AFLP fingerprint, indicating that no duplicates were present.

The genetic variability amongst the soursop accessions in the germplasm bank is relatively low, while the variability of several related Annona species is high even though the number of accessions is low.

The collection of more accessions and species is necessary in order to increase the variability of the germplasm bank.

AFLP molecular markers can be used for the identification of clones and future varieties of the analyzed species.

*4.2.2.3 References:*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### 4.2.3 *Anthracnose in Soursop (*Annona muricata*) in the Production Areas of Valle del Cauca, Colombia*

Tropical fruit crops comprise one of the better options of Colombian agriculture. National and international demand has been growing at 3.75% per year in the last 4 years, and the potential for activating a profitable postharvest industry (e.g., juices and jams) is high.

One promising tropical fruit is soursop (*Annona muricata*), for which demand is high and supplied by imports. Its organoleptic characteristics make it a promising fruit for fresh consumption and industrial markets, both national and foreign. The soursop crop generates high employment (250 working days per hectare per year), surpassing crops such as oil palm, sugarcane, or rice. It is also highly profitable (US$2500 per hectare per year in technicized crops).

However, because Colombia is the center of origin for soursop, the crop suffers various problems caused by phytopathogens that had co-evolved with it, in particular, anthracnose. This fungal disease, caused by *Colletotrichum* species, is spreading as the crop expands into areas that are environmentally favorable for the fungus. As a result, anthracnose is now a major constraint to soursop production in Colombia.

Our study aims to identify and characterize in morphological, genetic, and pathogenic terms *Colletotrichum* isolates obtained from soursop crops in Valle del Cauca, a major production area in southern Colombia.

#### *4.2.3.1 Materials and Methods*

**Sample collection**. Samples were obtained from leaves, branches, and flowers of soursop trees showing symptoms characteristic of anthracnose. Details of lesion type were recorded. Sampling included farms with different levels of disease incidence and located in the production areas of Valle del Cauca (Table 1).

**Isolating the pathogen** To discover the best methodology to disinfect, incubate, and isolate the pathogen associated with anthracnose, samples obtained in the first take were cut into fragments of healthy and diseased tissue. These fragments were submitted to the following disinfection procedures to promote growth and facilitate isolation of the pathogen: (1) washing with water for 1 h, 70% alcohol for 45 s, and water for 1 min; (2) washing with water for 1 h; and (3) washing with water for 10 min, 70% alcohol for 45 s, and water for 1 min. Isolates were obtained by either growing in the humidity chamber or planting in culture medium.

**Humidity chamber.** The disinfected fragments were placed in the humidity chamber on two glass slides in petri dishes containing damp, sterilized, paper toweling, and incubated at 22°C for 48 h. Once sporulated, the fungus was isolated on an artificial culture medium. The planted samples were incubated at 27°C in total darkness for 5 days, with periodic checks for contamination. After obtaining pure isolates, sporulation was stimulated by

scraping mycelium in the same petri dish and incubating again for 2 or 3 days.

**Table 1. Farms evaluated for anthracnose in soursop, Department of Valle del Cauca, Colombia**

| Farm | Municipality | Observations |
|---|---|---|
| Corporación BIOTEC experimental lot at CIAT station | Palmira | Some trees with leaf lesions |
| Agrícola Varaonda | Pradera | Some trees with lesions in leaves, branches, and flowers |
| Venecia | Santa Elena, El Cerrito | No disease found |
| Lot at CORPOICA Regional 5 | Palmira | High incidence of lesions in leaves and branches |
| La Marcela | Sevilla | Some trees with leaf lesions |
| San Rafael | La Union | Some trees with lesions in leaves and branches |
| Samaria | La Union-Toro | No disease found |
| La Esperanza | La Union | No disease found |
| Brasil | Anserma Nuevo | Some trees with leaf lesions. The lot was fumigated with cupric fungicide |
| Venecia 3 | Caicedonia | High incidence of disease |

***Planting in culture médium***

Five fragments of plant tissue of about 0.25 $cm^2$, previously disinfected by method (1) described above, were planted in petri dishes containing the following culture media: potato dextrose agar (39 g PDA/1 L of water), oat agar (OA; 10 g each of agar and oats per 1 L water), PDA + copper (85 mg Cu/L, using as source of copper, the fungicide Kocide®), and PDA + yeast extract (5 g/L) + peptone (5 g/L). To all the media, 10 mL/L of either 25% lactic acid (LA) or sptreptomycin 300mg/L were added after autoclaving the medium and cooling it to less than 45°C. The prepared petri dishes were incubated for 5 days at 27°C in darkness.

The samples taken in later collecting activities were processed only with the selected methodology.

**Monosporic cultures.** Spores were scraped from a pure isolate into water agar (20 g/L of water) and the whole planted as drops at a rate of 15 g/L into petri dishes containing fresh dextrose agar. The spores were then incubated for 24 h at 27°C in darkness, after which they were checked under a stereoscope for germination. The sites of individual germinated spores were marked on the agar with a micro-hook, and their presence confirmed, using a microscope and inverting the petri dish to observe the spore through the medium. To check the spore, an agar cube with the spore was cut out with the micro-hook and transferred to a new petri dish containing sterilized filter paper (Whatman® #1) on PDA + streptomycin at 300 mg/L (PDA + S). The spore was checked that it was in contact with the new medium. The petri dishes were incubated for 7 to 10 days at 27°C.

**Conserving the isolates.** The isolates were kept in petri dishes containing PDA medium, being transferred to fresh medium about every 3 weeks. For long-term conservation, the monosporic isolates were transferred to filter paper, following Girlena Aricapa's methodology (1994 CIAT, personal communication), but with the following modifications:

- Fragments of sterilized filter paper (Whatman® #1) of about 4 $cm^2$ were placed on PDA + S. Agar cubes, each with one germinated spore, were transferred to the paper. The spores were then left to incubate for 3 days at 27°C with alternate periods of 12 h of light and 12 h of darkness. Finally, the fungus was spread about in the same petri dish to accelerate colonization of the paper and again left to incubate for 5 to 7 days.
- Once completely colonized by the fungus, the paper fragments were removed from the medium and placed upside down in sterilized, empty, petri dishes. The part with mycelial growth and spores was placed on the petri dish's bottom so that the filter paper would not roll. The entire process was conducted in a laminar flow chamber. The prepared dish was immediately dried at 27°C for 7 days. Once dried, the isolates were stored in waxed paper envelopes (4 × 10 cm) at -20°C, each envelope being identified by a number assigned to the collection.

**Morphological characterization.** Colony morphology was evaluated for 56 isolates, which had been obtained from different production areas of Valle del Cauca and were growing on PDA + S medium. The isolates were then incubated at 29°C for 78 h. Four replicates per isolate were done, and the variables "colony diameter" and "mycelium color" evaluated.

**Pathogenicity trials.** To evaluate the pathogenicity of the *Colletotrichum* isolates, a trial was carried out in the greenhouse with the 56 isolates. Small trees that had been healthy as seedlings were inoculated. These trees were 3-month-old scions from the canopy of a tree of the soursop cultivar Elita, and grafted on a sexually produced individual indigenous to the region (produced by Profrutales S.A.). On being transferred to the greenhouse, the plants were left for 13 days to adapt to their new conditions and to strengthen their grafts.

**Inoculum preparation.** Each isolate was planted in the media PDA + S and OA + 300 mg streptomycin per liter, with five replicates per isolate (three petri dishes with PDA and two with OA), by scattering at random spores scraped from a pure monosporic culture onto the medium's surface. The isolates were then incubated for 15 days at 25°C, with alternate periods of light and darkness. Later, a suspension of spores was prepared by adding sterilized water directly onto the colony in the petri dish to resuspend the spore mass. This first suspension was collected in a 50-mL Falcon® tube, filtering with gauze to eliminate mycelium and medium fragments. (The number of petri dishes used per isolate depended on the abundance of spores.) The suspension was concentrated to 1X $10^7$ spores/mL, and Inex®, a dispersant for the spores, added to finally concentrate this product to 0.5%.

**Inoculation.** Before inoculation, all the trees, including the checks, had their old leaves removed before being sprayed with Carborundum® (an abrasive powder). To inoculate, a vacuum pump and DeVilbiss sprayer were used at distances of 30 cm between sprayer and plants. Each isolate was inoculated onto four soursop trees as follows:

- *By wounding*: small v-shaped grooves were cut into the stem at three sites, using disposable razor blades. A fragment of fungus was scraped directly onto the surface of each site from the petri dish colonized by the isolate.
- *By spraying:* with a spore suspension at 1X $10^7$/mL, and using a vacuum pump and a DeVilbiss sprayer, the entire plant was wetted with the suspension, concentrating on stem, apex, and youngest leaves (the first 6 from the apex).

For control, trees were sprayed with sterilized distilled water, and no inoculum was added to the three wounds made in each stem.

**Incubation conditions.** The inoculated plants were taken to a humidity chamber, placing them at random, and left for 72 h at 100% relative humidity and 27°C. The plants were then transferred to a greenhouse having an average temperature of 29°C. Here, they were wetted for 5 min every hour for 13 days, and then for 1 min, every 3 h until the trial was finalized. These conditions simulated rainy season conditions in Valle del Cauca.

**Evaluations.** A scale was designed to evaluate the disease, taking into account the appearance of lesions on the stem, which farmers consider as the key symptom causing most harm to the trees. The first evaluation was carried out at 72 h of continuous wetting; the second, 10 days after the first evaluation; and the third, 20 days afterward.

An *in vitro* methodology was developed to permit rapid evaluation of the isolates in terms of their capacity to reproduce foliar symptoms of the disease. Mature leaves and new shoots were collected from soursop trees, washed with deionized water, and left in fresh deionized water to prevent dehydration. Before inoculation, each leaf was disinfected with 70% alcohol and placed in a humidity chamber. For inoculation, small punctures in the leaf blade were made with a micro-needle, and 30 µL of the spore suspension, concentrated to$10^6$ spores/mL, placed on the holes. The humidity chambers were incubated to 30°C with alternate periods of 12 h of light and 12 h of darkness. Evaluations were made every 24 h until symptoms were observed.

**Sensitivity to benomyl.** The fungicide Benlate® (containing 50% benomyl as active ingredient) provokes differential response in some *Colletotrichum* species causing anthracnose in citrus fruits. Consequently, four isolates obtained from soursop trees and showing different morphological characteristics on media, were evaluated *in vitro* to determine response to benomyl. In the medium PDA + LA, modified with 2 µg/mL benomyl, disks of mycelial growth were planted at three replicates per isolate. Each disk measured 0.7 cm in diameter. A control was set up, consisting of disks of medium with no mycelial growth. Incubation conditions were 27°C, with alternate periods of light and darkness. The diameters of colonies were measured 48 h after the trial began. The trial was repeated to include the 56 isolates previously evaluated for morphology.

**Fungigram.** The efficiency of 25 chemical and 5 biofungicides in inhibiting the growth of *Colletotrichum* spp. was evaluated (**Table 2**). Three isolates were selected based on colony morphology and growth rate, 20451 characterized by slow growth and dark gray mycelium, m3612sr a white mycelium, and 17m3612sr a fast growing colony with grayish-white mycelium, were selected from the collection . In petri dishes, 100 µL of a spore suspension

from the isolates, concentrated to 1X $10^6$ spores/mL, were spread over PDA + LA medium. Disks of sterilized filter paper, 6 mm in diameter, were impregnated with a solution of each product to be evaluated, placing four disks at random in each dish, where the fungus had previously been planted. As control, disks moistened in sterilized distilled water were used. The dishes were incubated at 27°C. The treatments were replicated three times, under a randomized complete block design.

The effectiveness of the chemical products was assessed by measuring the diameter of the area not colonized by the fungus around the disk, which would correspond to the inhibiting effect of the product on the pathogen. The evaluations were made from the third day onward.

**DNA extraction.**

The biomass production (mycelium) for DNA extraction was made in a liquid medium, PDA broth (infusion of 200 g potato and 20 g dextrose per liter of water). Each isolate was planted and incubated for 15 days at 25°C with alternate periods of 12 h of light and 12 h of darkness. The source of the isolate for planting was a culture grown on PDA + S medium. Once the period of growth in the liquid medium was completed, the medium was filtered through sterilized filter paper to catch the mycelium, which was then placed in petri dishes on sterilized filter paper and dried at 35°C for 3 days. It was then macerated with liquid nitrogen and deposited in 1.5-mL Eppendorf tubes, using 0.3 g of dried macerated mycelium per tube to initiate extraction. The remaining dried mycelium was stored in Eppendorf tubes at -20°C.

Following George Mahuku's protocol, 2002, Bean Phytopathology, CIAT, with some modifications 0.3 g of dried macerated mycelium were placed in a 1.5-mL Eppendorf tube, and a 900-µL volume of extraction buffer added (1.4 M NaCl, 20 mM EDTA, and 100 mM Tris-HCl pH 8.0), together with 1.5 µL of proteinase K at 10 mg/mL. The whole was vortex mixed to obtain homogeneity.

The mixture was incubated at 65°C for 1 h. Then, 200 µL of 7.5 M ammonium acetate were added and the whole left at room temperature for 10 min. The mixture was then centrifuged at 12,500 rpm for 20 min, and the supernatant transferred to another 1.5-mL Eppendorf tube, to which was added an equal volume of chloroform:isoamyl alcohol (24:1).

The whole was mixed by inversion and centrifuged at 12,500 rpm for 10 min to recover the supernatant. This was transferred to a new 1.5-mL Eppendorf tube and one-third the volume of cold isopropanol alcohol added. The whole was kept at -20°C overnight or for at least 2 h, and again centrifuged at 12,500 rpm for 10 min.

**Table 2.** Chemical and biofungicides evaluated for the control of *Colletotrichum* isolates, as demonstrated by the diameter of the inhibition halo.

| *Fungicide* | | Dose (per liter) | Type of action | Inhibition halo (cm) |
|---|---|---|---|---|
| **Trade name** | **Active ingredient** | | | |
| Control | Chlorothalonil (Chlortocaffaro®) | 2-5 g | Contact | 0.9 |
| Euparen WP50 | Dichlofluanid | 2-3 g | Protectant | 1.3 |
| Sandofan | Oxadixyl + mancozeb | 1.6 g | Systemic protectant | 0.6 |
| Orthocide 50% | Captan | 2.5 g | Protectant | 0.8 |
| Score 250 E | Difenoconazole | 0.6 cc | Systemic | 1.7 |
| Anvil 5% SC | Hexaconazole | 1.2 cc | Systemic, preventive, curative, eradicative | 1.4 |
| Fungi-bact SL | Benzalkonium chloride | 1-1.2 cc | | 0.0 |
| Molto EC (NA) | Propiconazole + prochloraz | 20 cc | Systemic | 2.8 |
| Mertec 500 SC | Thiabendazole | 0.9 cc | Systemic | 1.7 |
| Tilt 250 EC | Propiconazole | 0.2 cc | Systemic | 0.0 |
| Saprol DC | Triforine | 1.25 cc | Systemic translaminar | 0.0 |
| Alto | Cyproconazole | 0.5 cc | Systemic | 0.0 |
| Previcur | Propamocarb hydrochloride | 1.5 cc | Systemic, curative, protective | 0.0 |
| Derosal | Carbendazin | 1.5 cc | Systemic, preventive, curative | 0.0 |

| | | | | |
|---|---|---|---|---|
| Microthiol | Sulfur 80% | 1.0 g | Protectant, eradicant | 0.0 |
| Ridomil | Metalaxyl | 3.0 g | Systemic protectant | 0.0 |
| Octave | Prochloraz | 1.0 g | Preventive, curative translaminar | 2.0 |
| Cobrethane | Mancozeb + copper oxychloride | 2.5 g | Preventive | 0.0 |
| Rovral | Iprodione | 1-1.5 g | Protectant | 0.0 |
| Sialex | | 1.0 g | | 0.0 |
| Trifmine | Triflumizole | 1.0 cc | Curative, preventive | 1.8 |
| Swinglea 5% | | | | 0.0 |
| Aliette | | 3.0 g | | 0.0 |
| Dithane M-45 | | | | 0.0 |
| Vitavax | | 2.0 g | | 0.0 |
| ***Plant extract*** | | | | 0.0 |
| Horsetail plant (Equisetum arvense) | | 5% | | 0.0 |
| Wild pinguin 5% (Bromelia karatas) | | 5% | | 0.0 |
| Salvia 5% | | 5% | | 0.0 |
| Lixiviated lime | | | | 0.0 |
| Lixiviated plantain | | | | 0.0 |
| Sterilized distilled water (control) | | | | 0.0 |

The supernatant was discarded, leaving a pellet, which was washed by adding 350 µL of cold 70% ethanol while gently tapping the tube to resuspend the pellet. The whole was again centrifuged at 12,000 rpm for 5 min. This washing procedure was repeated twice, with the supernatant discarded each time. The pellet remaining on the bottom of the tube was left to dry to eliminate excess alcohol before being resuspended in 50 µL of TE buffer.

With monosporic cultures in solid media, trials for extraction and direct amplification of DNA were carried out, starting with spores, to seek an alternative that would facilitate identification of the pathogen.

Three protocols of DNA extraction from spores were followed:

**Dolye and Dolye's protocol (1987), with modifications.** In a 2.0-mL Eppendorf tube, a volume of about 0.3 cc of spores produced on solid medium was kept at -80°C for 5 min. Then, 50 µL of TE buffer were added, the whole heated in a microwave oven for 30 s. Then, 1 mL of CTAB extraction buffer (2% CTAB pp/v, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 1% PVP, and 1% mercaptoethanol) and 1/10 volume of phenol:chloroform:isoamyl alcohol (25:24:1) were added and vortex mixed to obtain homogeneity. The tubes were incubated at 65°C for 30 min. They were then centrifuged at 12,000 rpm for 20 min.

The supernatant was transferred to another 2.0-mL Eppendorf tube, to which was added an equal volume of chloroform:isoamyl alcohol (24:1), mixed by inversion and centrifuged at 12,000 rpm for 10 min, and the supernatant recovered. This last was transferred to 1.5-mL Eppendorf tubes, and about one-third volume of cold isopropanol alcohol added. The whole was kept at -20°C overnight or for a minimum of 1 h. The supernatant was then centrifuged at 12,000 rpm for 20 min and the new supernatant discarded. Cold 70% ethanol (350 µL) was added, tapping gently to resuspend the pellet, and the whole centrifuged for 5 min at 12,000 rpm. The washing was repeated twice, and the supernatant discarded each time, leaving the pellet at the bottom of the tube. The pellet was left to dry to eliminate excess alcohol and resuspended in 50 µL of TE buffer.

To use the RNAse, the pellet was resuspended in 200 mL of ultra-pure water and 4 µL of RNAse added. The whole was left to incubate for 2 h at 37°C, after which an equal volume of chloroform:isoamyl alcohol (24:1) was added and the whole centrifuged at 4°C for 10 min at 12,000 rpm. The supernatant was recovered, and 1/10 volume of 3 M sodium acetate (pH = 7.0) and an equal volume of cold isopropanol alcohol were added. The whole was kept at -20°C overnight or for a minimum of 1 h. The supernatant was then centrifuged at 12,000 rpm for 20 min and the new supernatant discarded. To wash, 350 µL of cold 70% ethanol were added, tapping gently to resuspend the pellet. The whole was then centrifuged and the new supernatant discarded, leaving the pellet at the bottom of the tube. The pellet was left to dry to eliminate excess alcohol and resuspended in 50 µL in TE buffer.

**George Mahuku's protocol, with modifications (2002, personal communication).** A volume of about 0.3 cc of spores produced on solid medium was added to a 2.0-mL Eppendorf tube and kept at -80°C for 5 min. Then, 50 µL of TE buffer were added, heated

in a microwave oven for 30 s, and 800 µL of CTAB extraction buffer (2% CTAB pp/v, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 1% PVP, and 1% mercaptoethanol) and 1.5 µL of proteinase K were added and mixed by inversion to obtain homogeneity. The tubes were then incubated for 1 h at 65°C. Then 200 µL of 7.5 M ammonium acetate were added and left for 10 min at room temperature before being centrifuged for 20 min at 12,000 rpm.

The supernatant was transferred to another 2.0-mL Eppendorf tube to which an equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed by inversion before centrifuging at 12,000 rpm for 10 min to recover the supernatant. This last was transferred to 1.5-mL Eppendorf tubes, 0.3 vol of cold isopropanol alcohol added, and the whole left at -20°C overnight or for at least 1 h. The supernatant was then centrifuged at 12,000 rpm for 20 min and the new supernatant discarded. To wash, 350 µL of cold 70% ethanol were added, tapping gently to resuspend the pellet, which was then centrifuged for 5 min at 12,000 rpm. The washing was repeated twice, with the new supernatant being discarded each time, leaving a pellet at the bottom of the tube. The pellet was left to dry to eliminate excess alcohol before being resuspended in 50 µL of TE buffer.

**Using Chelex-100- protocol (Alvarez E. 2002).** About 1 volume of 0.3 cc of conidia was collected in a 1.5-mL Eppendorf tube. Then, 400 µL of Chelex-100, prepared to 5% in sterilized water, were added to each sample and gently vortex mixed for 3 s. The samples were incubated at 56°C for 2 h. Each sample was then vigorously vortex mixed for 3 s and incubated over a bain-marie at 98°C for 8 min. The whole was vortex mixed again for 3 s and centrifuged at 15,000 rpm for 5 min. Finally, the supernatant containing DNA was transferred to another tube and the pellet discarded.

For the extractions with the above protocols, the spores were previously macerated with sand. To confirm the quality of the DNA, it was run in a 1% agarose gel stained with 72 ng ethidium bromide per mL of gel.

**Amplifying the ITS region.** To identify those *Colletotrichum* species associated with anthracnose in soursop and to determine their genetic variability, the ITS region was amplified through PCR, using the universal primers ITS 1(TCCGTAGGTGAACCTGCGG) and ITS 4(TCCTCCGCTTATTGATATGC), and ITS 1 and ITS 2 (GCTGCGTTCTTCATCGATGC), and the specific primers CgInt (GGCCTCCCGCCTCCGGGCGG) and CaInt2 (GGGGAAGCCTCTCGCGG), each in combination with primer ITS 4. The ITS (internal transcribed spacer) region is found intercalated among genes that code for ribosomal subunits and, because of the facility with which sequences of the spacers can be obtained and interpreted, the ITS region is widely used to study the molecular phylogeny of plants and microorganisms. Moreover, its small size, highly conserved flanks, large number of copies, and fast concerted evolution facilitate analysis through polymerase chain reaction (PCR), sequencing, alignment, and phylogenetic analysis, thus permitting discrimination of genera and species.

For each PCR reaction, we used 10X *Taq* buffer at a final concentration of 1X/µL (100 mM Tris-HCl pH 8, 2.5 mM $MgCl_2$ , and 500 mM KCl), 0.2 mM of each dNTP, 0.5 mM of each primer, 1.5 mM $MgCl_2$, 2 ng/µL DNA, ultra-pure water, and 0.0375 U/µL *Taq* polymerase.

Conditions for amplification were standardized by means of preliminary trials, using a thermocycler (PTC-100, MJ Research, Watertown, MA).

Cycling conditions for DNA amplification were an initial denaturation of 94°C for 2 min. and 40 cycles of 30 s. at 94°C (denaturation) 30s at 55 (annealing), 2 min at 72°C ( extension) and a final extension of 4 min at 72°C.

**Restriction with enzymes.** To analyze restriction enzymes, 10 µL of the product amplified by PCR with primers ITS 1 and ITS 4, were used, together with 2 µL of 10X buffer for the enzyme, and 0.6 µL of the enzyme at a concentration of 10,000 U/µL. When required, 0.2 µL BSA was added to the cocktail. The final volume of the reaction was 20 µL when made up with ultra-pure water. The digestion reaction was incubated at the temperature specific to each enzyme.

Initially, 10 isolates with different morphological characteristics were analyzed. Those restriction enzymes showing polymorphism in the cutting patterns among these isolates were used to cut the amplified product of the remaining 46 isolates.

**Random amplified microsatellites.** To determine the genetic variability of *Colletotrichum* isolates, random amplification was used with RAMS primers. For the PCR reaction, we used 10X *Taq* buffer to a final concentration of 1X/µL (100 mM Tris-HCl pH 8, 2.5 mM $MgCl_2$, and 500 mM KCl), 0.2 mM of each dNTP, 1 mM of each primer, 1.5 mM $MgCl_2$, 1 ng/µL DNA, ultra-pure water, and 0.0375 U/µL *Taq* polymerase. The RAMS primers are repetitions-in-tandem of sequences of two or three nucleotides with random bases at the 3' end, with which the genetic variability of individuals belonging to closely related gene pools can be estimated, permitting differentiation between species and within species, according to the amplification patterns from the total DNA. The polymorphism obtained with the different RAMS primers on seven *Colletotrichum* isolates was evaluated to then amplify the 56 isolates with the most polymorphic primer.

**Electrophoresis in agarose gels.** To visualize and analyze the amplification products, electrophoresis was carried out in 1.5% or 2% agarose gels containing 0.5 µg/mL ethidium bromide and 0.5X TBE buffer (Trisma base, boric acid, and EDTA). Conditions for electrophoresis were 100 volts and 35 milliamperes.

To visualize the total DNA, electrophoresis was carried out in 0.8% agarose gels with 0.5 µg/mL ethidium bromide and 0.5X TBE buffer. Conditions for electrophoresis were 70 volts and 15 milliamperes. Digestion with restriction enzymes was run in gels of greater resolution: 0.7% agarose and 0.5% Sinergel®, with 0.5 µg/mL ethidium bromide and 0.5X TBE buffer. These were run at 120 volts 65 milliamperes. Run time depended on gel size.

*4.2.3.2 Results and Discussion*

**Sampling.** In the initial samplings, low incidence of disease was observed in commercial crops and in non-commercial, household trees, mainly because climatic conditions were dry. Interviews with farmers confirmed that severe outbreaks of the disease occur in rainy seasons, when management of the disease—understood by them as chemical control—

becomes important. Sampling after the rains carried out on most farms confirmed that the climatic conditions of rainy seasons favor disease development in terms of dynamics and epidemiology.

**Symptoms.** The samplings permitted the definition of typical symptomatology of the disease (**Figure 1**). In leaves, lesions were dark, necrotic, with a darker margin, no well-defined chlorotic halo nor specific form, but a well-defined front of advance. The lesions were located mainly in the leaf apex, advancing toward the peduncle, covering the width of the leaf blade, and sometimes producing crinkling when lesions located on the network of veins on the leaf's upper surface were larger than those on the lower surface.

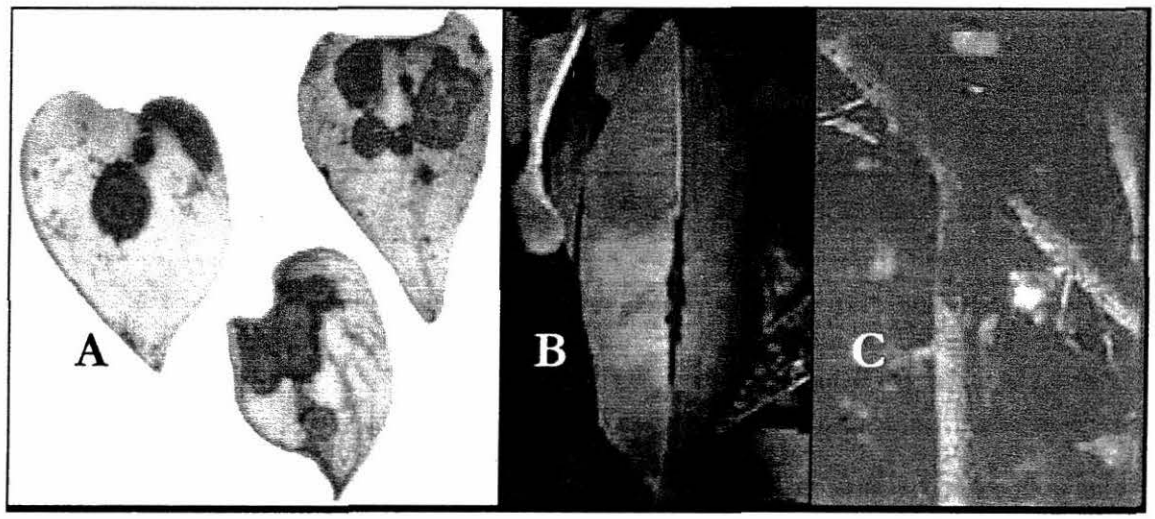


**Figure 1.** Symptoms characteristic of anthracnose in soursop: (A) flowers; (B) leaves; (C) branches

In flowers, lesions were located mainly on the sepals, and were dark, rounded, well defined, and causing depressions. On stems and branches, lesions were black spots, initially oval or round, with irregular margins and looking like burns. They caused depressions in the bark at their sites. When we longitudinally cut open a lesion on an affected branch, a very dark necrotic process could be observed, looking like carbonized tissue. If the lesion is well established, a form of crust can be seen on the bark at the site of the "burn".

**Isolating the pathogen and its morphological characterization.** From sampling carried out in six areas of Valle del Cauca and Eje Cafetero (also southern Colombia), 109 isolates were obtained (**Table 3),** which were stored on filter paper at -20°C, and conserved in Eppendorf tubes containing the medium PDA at 4°C.

**Table 3.** Origin of samples and number of *Colletotrichum* isolates obtained in Valle del Cauca and Quindío, Colombia.

| Origin | Isolates (no.) |
|---|---|
| Anserma Nuevo | 3 |
| Caicedonia | 34 |
| CIAT, Cali | 11 |
| CORPOICA, Palmira | 12 |
| La Unión | 13 |
| Quindío | 1 |
| Pradera | 34 |
| Bitaco | 1 |
| **Total** | **109** |

The pathogen was effectively isolated, even from initial lesions, following disinfection method (1), based on washing with water for 1 h, disinfection in alcohol for 45 s, and final washing in water for 1 min. The samples were incubated in a humidity chamber to promote sporulation.

The collection of isolates showed four colony types (**Figure 2**). Colony type A was characterized by very slow growth, a gray mycelium, and slow sparse sporulation with an undulating appearance. This type of colony was frequently obtained from lesions in very young leaves. Type B was characterized by rapid growth, sparse white mycelium, and fast, abundant, and orange-colored sporulation. Type C was also fast growing, with grayish-white mycelium that was abundant, spongy and, on agglomerating, generated a dark compact tissue. Sporulation was moderately abundant, with well-defined, drop-like, brilliant orange-colored masses of spores. Type D grew rapidly, with abundant gray mycelium, and very little sporulation. The type of colony detected most frequently was A (61%), followed by C (29%), B (9%), and D (2%).

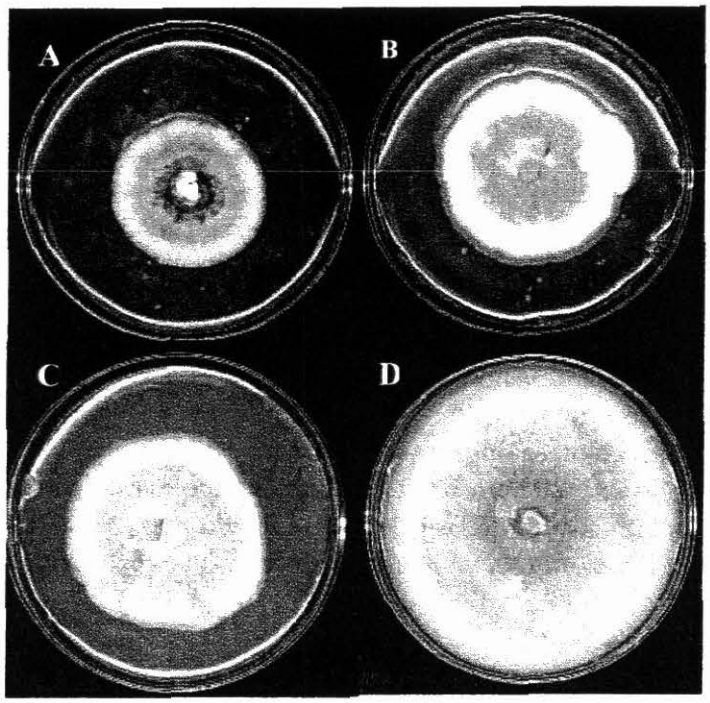


**Figure 2.** Types of colonies of *Colletotrichum* isolates obtained on medium PDA + S. Isolates: A = 19943; B = m13sr rosa; C = m26; D = m13sra.

**Figure 3** shows a separation of isolates according to the average diameter of their colonies 72 h after planting in medium PDA + S. No isolates with colony diameters between 2.6 and 3.0 cm were found. Isolates with colony diameters smaller than 2.6 cm represented colony types A and B, whereas isolates with diameters larger than 3.0 cm were of type C.

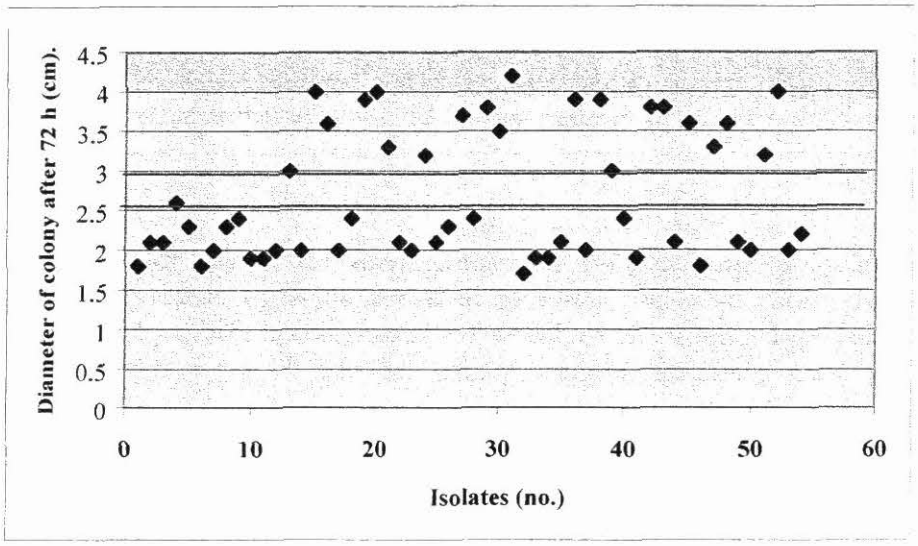


**Figure 3.** Colony diameters of 56 *Colletotrichum* isolates on medium PDA + S, as reported in Table 5.

**Pathogenicity trials.** Taking into account the field and greenhouse observations and farmers' experiences, a scale for evaluating the disease was designed, based on the appearance of lesions in stems—the symptom considered as causing the most harm to soursop trees. These lesions are defined as initially oval or round black spots, with irregular margins, looking like burns, depressing the bark in the spots' sites, and having no chlorotic margins. The scale, from 0 to 5, is as follows (**Figure 4**):

- 0 = total absence of lesions on stems;
- 1 = at least one lesion on stems
- 2 = several small lesions along the stem's length
- 3 = several small and medium-sized lesions along the stem's length
- 4 = small, medium-sized, and large lesions along the stem's length
- 5 = total plant deterioration, with lesions coalescing so that their size

  cannot be easily distinguished

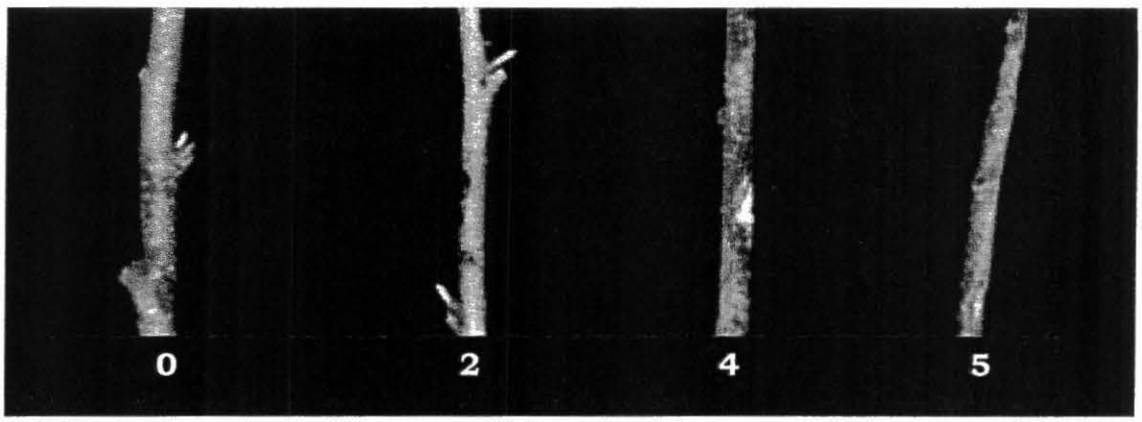


**Figure 4.** Evaluation scale for damage caused by *Colletotrichum* spp. to the soursop cultivar Elita.

The symptomatology produced on inoculating stems was similar to that observed in the field. Typical lesions were also observed in leaves, although evaluation was hindered by defoliation, making it impossible to follow up foliar lesions. Although defoliation was not consistent between replicates, infected plants scoring 3 and 5 showed heavy or total defoliation.

The data obtained with this trial were analyzed statistically, using the SAS program. To better understand the damage caused, the area under the disease progress curve (AUDPC) was calculated for each isolate throughout the experiment. The data for each evaluation and colony diameters were analyzed separately for each isolate.

From the second evaluation onward, the analysis of variance (**Table 4**) showed significant

differences between isolates with regard to pathogenicity. The first evaluation had been carried out only 4 days after inoculation, which was insufficient time for infection to develop or symptoms to appear.

**Table 4.** [a]Analysis of variance (ANOVA) for pathogenicity of 56 isolates of *Colletotrichum* obtained from Valle del Cauca Colombia and inoculated on soursop [c]cultivar Elita

| *Source of variation* | Df | Pathogenicity[b] (mean square) | Pr > F |
|---|---|---|---|
| Isolates | 58 | 4758.9619** | 0.0001 |
| Coefficient of variation % | | 40.89736 | |

[a]. ANOVA was performed on a single experiment; 59 treatments were inoculated in a soursop cultivar, arranged in a randomized complete block design experiment with four plants by treatments and 4 replicates; 3 controls (inoculated with water).

[b] Mean square obtained by area under disease progress curve (AUDPC)

** and NS represent significant effects at the $P \leq 0.01$ and no significance at $P<0.05$ respectively.

[c]. Soursop cultivar: Elita

Of the 56 isolates evaluated, 7.7% did not reproduce symptoms in inoculated plants; 50.9% reproduced symptoms scoring more than 0, but less than 3 on the evaluation scale; and 41.8% reproduced symptoms scoring 3 or more.

The most aggressive isolates were slow growing on medium PDA + S. The correlation coefficient between the variable colony diameter and pathogenicity (AUDPC) was -0.84.

When the isolate 'bitaco' was inoculated by spraying (but no wounding), the symptoms were observed to appear in the stem, suggesting that, under high inoculum pressure, spraying is sufficient to bring on symptoms.

**Sensitivity to benomyl.** Of the 56 isolates evaluated for their sensitivity to benomyl, 55 showed no growth. Only the control isolate obtained from cassava and identified as *C. acutatum* grew in the medium with benomyl. Eight days after the experiment began, a few isolates showed some growth of hyphae submerged in the medium. In the trial, carried out with four morphologically different isolates, isolates 19943 and m13srb showed tolerance of benomyl, growing in PDA + benomyl. Isolates m13sra and m13sr rosa did not grow in this medium, and are considered sensitive to benomyl. Figure 5 shows benomyl sensitivity in two isolates.

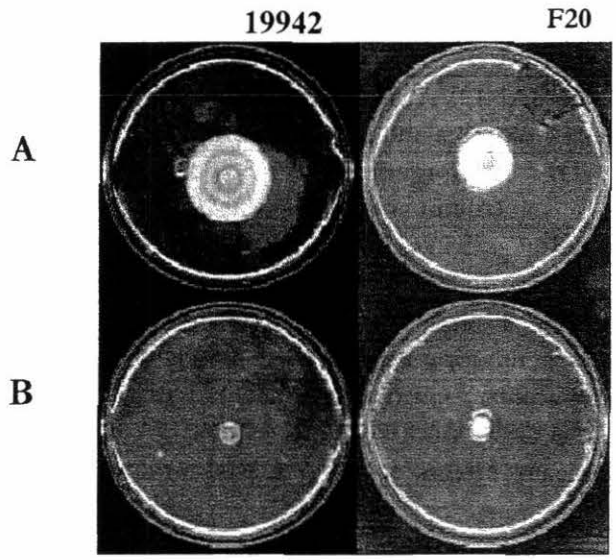


**Figure 5.** *Colletotrichum* isolates 19942 and f20 evaluated for their sensitivity to benomyl. A) Control (PDA + Al), with no benomyl; (B) treatment (PDA + Alb + benomyl).

**Fungigram.** Of the 30 fungicides evaluated, 3 showed control, generating a halo of growth inhibition around the *Colletotrichum* isolates (**Figure 6**). The product showing the best control was Molto®, (4)which showed the largest inhibition halo (at 2.8 cm), 72 h after the trial began.

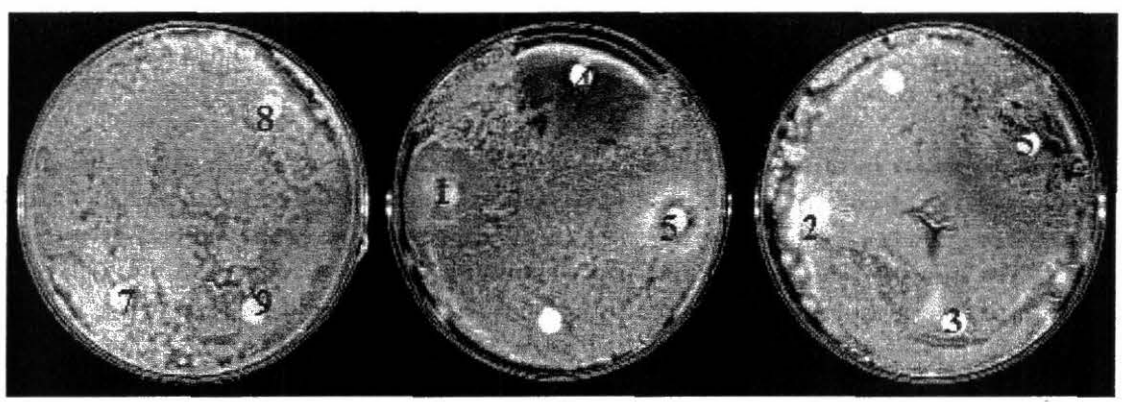


**Figure 6**. Inhibition halos of some trade mark fungicides controlling a *Colletotrichum* isolate (20451): (1) Octave; (2) Fungi-bact SL; (3) Tilt 250 EC; (4) Molto; (5) Score; (7) Derosal; (8) Previcur; (9) Microthiol.

Molto® is a mixture of two active ingredients—prochloraz and propiconazole—that separately presents a different action on the pathogen. The propiconazole, also the active ingredient of Tilt®, does not generate inhibition, whereas prochloraz, the active ingredient of Octave®, generates an inhibition halo of 2.0 cm at 72 h. These findings suggest that the active ingredient controlling the pathogen is really prochloraz, and that, when in

combination with propiconazole, its action is strengthened, as reflected by a larger inhibition halo.

The plant extracts were evaluated as clean alternatives of disease control, but they did not inhibit fungal growth.

**DNA extraction from mycelium.** Following the protocol described by G. Mahuku with some modifications, good quality DNA was obtained from 56 monosporic isolates.

**DNA extraction from spores.** The product from the DNA extracted from spores, using the modified protocols described by Dolye and Dolye, G. Mahuku, and J. L. Claroz, was observed in 1% agarose gel, stained with 72 ng ethidium bromide per mL of gel. Results were negative.

**Amplification of ITS regions.** The product amplified with primers ITS 1 and ITS 4 from the 56 *Colletotrichum* isolates associated with anthracnose in soursop was 550 bp long, whereas the product amplified with primers ITS 1 and ITS 2 was 240 bp long (**Figure 7**). Neither of the two combinations of primers showed polymorphism between isolates.

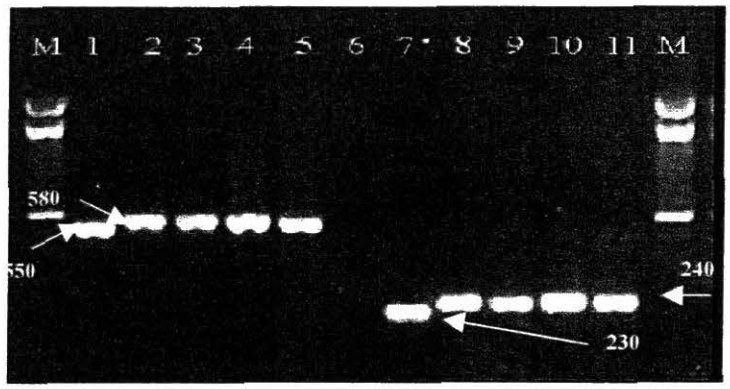


**Figure 7.** Amplified ITS region of ribosomal DNA from *Colletotrichum* isolates. M = 100-bp marker; lanes 1 and 7 = DNA from *Phaeoisariopsis griseola;* lanes 2 to 5 = product amplified with primers ITS 1 and ITS 4; lane 6 = negative control; lanes 8 to 11 = product amplified with primers ITS 1 and ITS 2.

**Amplification of ribosomal DNA with specific primers.** Based on 56 *Colletotrichum* isolates obtained from infected soursop tissue, 450-bp-long fragments were amplified with the combination of primers CgInt (specific for *C. gloeosporioides*) and ITS 4, as was the fragment amplified for the control isolate (*C. gloeosporioides* of citrus fruits) (**Figure 8**). In contrast, the PCR reaction for the specific primers CaInt2 (for *C. acutatum*) and ITS 4 amplified a fragment of genomic DNA of only 490 bp in five isolates, including the control (*C. acutatum* from cassava) (**Figure 9**). Of the evaluated isolates, 89.3% corresponded to *C. gloeosporioides* and 10% to *C. acutatum* (**Table 5).**

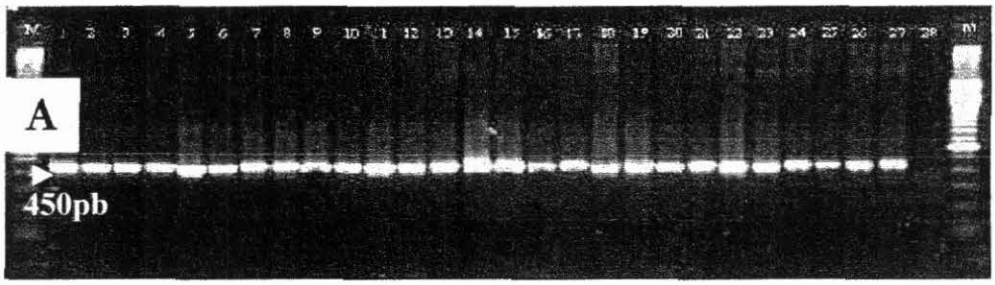


**Figure 8.** Amplification of specific fragments of ribosomal DNA from *Colletotrichum* isolates, using the primers CgInt and ITS 4. M = 100-bp marker; lane 1 = positive control *C. gloeosporioides*; lanes 2 to 27 = *Colletotrichum* isolates; lane 28 = negative control

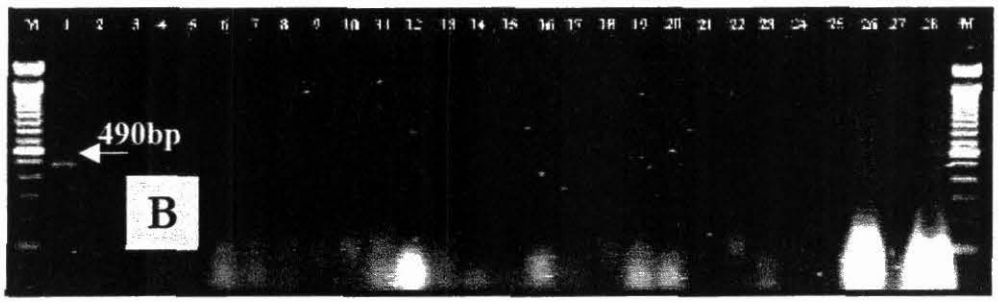


**Figure 9**. Amplification of specific fragments of ribosomal DNA from *Colletotrichum* spp., using primers CaInt and ITS 4. Lane 1 = positive control *C. acutatum*; lanes 2 to 27 = *Colletotrichum* isolates; lane 28 = negative control.

**Digestion of amplified products.** Digestion with restriction enzymes was carried out only on the product amplified with the primers ITS 1 and ITS 4, because the product amplified with the primers ITS 1 and ITS 2 was very small, and its digestion would have generated fragments that could not have been observed in agarose gels. Also, the probability of the restriction enzymes' cutting such small fragments would have been very low.

Enzymes *Rsa*I, *Dra*I, *Hae*III, and *Hind*III were first evaluated in 10 *Colletotrichum* isolates (**Figure 10**). No significant differences were seen in the restriction products of *Msp*1 (Figure 10A), generating fragments 290 and 110 bp long; and *Alu*I (Figure 10B), with fragments 280 and 95 bp long. Enzymes *Dra*I and *Hind*III (Figures 10D and 10E, respectively) did not have cutting sites, and enzymes *Hae*III and *Rsa*I (Figures 10C and 10F, respectively) showed differences among isolates. *Hae*III generated fragments 290, 180, and 105 bp long for all isolates. Enzyme *Rsa*I generated fragments 190 and 380 bp long for certain isolates or did not cut in others, creating two groups corresponding to two species.

**Table 5.** Origin of 58 isolates of *Colletotrichum* , and pathogenicity of these isolates to one cultivar of soursop (*Annona muricata*), Elita in Valle del Cauca Colombia.

| Identification | Site | Tissue | Diameter (cm) Average[a] | Pathog'y AUDPC[b] | Pathog'y group[c] | Diameter group[d] | Colony type[e] | RAMS group[f] | RFLP group[g] | CgInt and ITS 4[h] | CaInt2 and ITS 4[i] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 19942-40 | Anserma | Leaf | 1.8 | 98.25 | 5 | 1 | 1 | 1 | 1 | + | - |
| 19945-36 | Anserma | Leaf | 2.3 | 60.63 | 4 | 4 | 1 | 1 | 2 | + | - |
| 19945-35 | Anserma Nuevo | Leaf | 2.1 | 63.75 | 4 | 1 | 1 | 1 | 1 | + | - |
| 19945ped-36 | Bitaco | Peduncle | 2.2 | 80 | 5 | 1 | 1 | 2 | 2 | + | - |
| cd12rb-29 | Caicedonia | Branch | 3.6 | 27.25 | 3 | 3 | 3 | 2 | 2 | - | + |
| cd14r-42 | Caicedonia | Branch | 3.9 | 27.33 | 3 | 5 | 3 | 2 | 2 | + | - |
| cd23r-44 | Caicedonia | Branch | 3 | 14.5 | 2 | 2 | 3 | 2 | 1 | + | + |
| 19945-38 | Caicedonia | Leaf | 2 | 65.88 | 4 | 1 | 1 | 3 | 2 | + | - |
| 20451-15 | Caicedonia | Leaf | 2 | 69.75 | 4 | 1 | 1 | 2 | 2 | + | - |
| amonmt6h-3 | Caicedonia | Leaf | 3.6 | 11.75 | 2 | 3 | 3 | 3 | 2 | + | - |
| amonvs1ab2m6-4 | Caicedonia | Leaf | 3.3 | 10.75 | 2 | 2 | 3 | 3 | 2 | + | - |
| c1-19 | Caicedonia | Leaf | 4 | 2 | 1 | 5 | 3 | 3 | 2 | + | - |
| c5-26 | Caicedonia | Leaf | 3.8 | 2 | 1 | 5 | 3 | 3 | 2 | + | - |
| cd14h | Caicedonia | Leaf | 2 | 88.25 | 5 | 1 | 1 | 1 | 2 | + | - |
| cd1h | Caicedonia | Leaf | 2.1 | 87.13 | 5 | 1 | 1 | 2 | 1 | + | - |
| 20172ped | Caicedonia | Peduncle | 2.1 | 66.5 | 4 | 1 | 1 | 1 | 1 | + | - |
| cd23ra-44 | CIAT | Branch | 4 | 10 | 2 | 5 | 3 | 3 | 2 | + | - |
| cd40-41 | CIAT | Branch | 3.3 | 2 | 1 | 2 | 3 | 2 | 2 | + | - |
| cd43r | CIAT | Branch | 1.8 | 94 | 5 | 1 | 1 | 2 | 1 | + | - |
| cd6r-28 | CIAT | Branch | 3.4 | 25.25 | 3 | 2 | 3 | 3 | 1 | + | - |
| cd7r | CIAT | Branch | 2 | 71.5 | 4 | 1 | 1 | 2 | 1 | + | - |
| m10pdr-49 | CIAT | Branch | 2.3 | 31.75 | 3 | 4 | 1 | 2 | 2 | + | - |
| f20-12 | CIAT | Leaf | 3.2 | 20.5 | 3 | 2 | 3 | 3 | 2 | + | - |
| f21-5 | CIAT | Leaf | 3.8 | - | 1 | 5 | 3 | 3 | 2 | + | - |
| f28-17 | CIAT | Leaf | 3.6 | 2 | 1 | 3 | 3 | 3 | 1 | + | - |
| m17pdfl-37 | Corpoica | Flower | 2.1 | 11.67 | 2 | 1 | 1 | 3 | 3 | + | - |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| m12sra-9 | Corpoica | Leaf | 3.8 | 29.88 | 3 | 5 | 3 | 3 | 1 | + | - |
| m13pd | Corpoica | Leaf | 2.1 | 72 | 4 | 1 | 1 | 1 | 1 | + | - |
| m13sra-22 | Corpoica | Leaf | 3.7 | - | 1 | 3 | 3 | 3 | 2 | + | - |
| m13srb-31 | Corpoica | Leaf | 2.4 | 83.88 | 5 | 4 | 1 | 2 | 1 | - | - |
| m14pdh | Corpoica | Leaf | 2.6 | 55.75 | 4 | 4 | 1 | 2 | 2 | + | - |
| m15pd-47 | Corpoica | Leaf | 2.1 | 76.13 | 4 | 1 | 1 | 2 | 2 | + | - |
| cd12-43 | Corpoica | Leaf | 3.9 | 12 | 2 | 5 | 3 | 3 | 1 | + | - |
| m16a-34 | Corpoica | Leaf | 4 | 20.25 | 3 | 5 | 3 | 3 | 2 | + | - |
| m20pd | Corpoica | Leaf | 2.3 | 61.5 | 4 | 4 | 1 | 2 | 3 | + | - |
| m25pdrb | La Unión | Branch | 3.2 | 35 | 3 | 2 | 3 | 2 | 3 | + | - |
| m25pdfl-50 | La Unión | Flower | 1.9 | 87.75 | 5 | 1 | 1 | 2 | 1 | + | - |
| m26pdh | La Unión | Leaf | 1.9 | 73.13 | 4 | 1 | 1 | 2 | 1 | + | - |

**Tabla 5**. Continued

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| m3612b | La Unión | Leaf | 2.4 | - | 1 | 4 | 2 | 2 | 3 | + | - |
| m3612sr-46 | La Unión | Leaf | 3.5 | 13 | 2 | 3 | 2 | 3 | 1 | + | - |
| m3612srcg-18 | La Unión | Leaf | 2.4 | 11.75 | 2 | 4 | 2 | 3 | 3 | + | - |
| m3913sr-30 | La Unión | Leaf | 2.4 | 37.5 | 3 | 4 | 2 | 2 | 1 | - | - |
| m3brasil b-39 | La Unión | Leaf | 1.7 | 81.25 | 5 | 1 | 1 | 2 | 1 | - | - |
| m9pdr-45 | Pradera | Branch | 1.9 | 48.25 | 3 | 1 | 1 | 2 | 3 | + | - |
| m9pdra | Pradera | Branch | 2 | - | 1 | 5 | 1 | 2 | 1 | + | - |
| m3brasil-33 | Pradera | Leaf | 1.9 | 84.67 | 5 | 1 | 1 | 2 | 1 | + | - |
| m8brasila-35 | Pradera | Leaf | 2 | 74.75 | 4 | 1 | 1 | 2 | 3 | | - |
| mamt4caliz-25 | Pradera | Leaf | 4.2 | 6 | 1 | 5 | 3 | 3 | 1 | - | + |
| qh1m1-24 | Pradera | Leaf | 3 | 9.75 | 2 | 2 | 3 | 3 | 1 | + | - |
| 20171 | Pradera | Leaf | 2 | 107.25 | 5 | 1 | 1 | 2 | 3 | + | - |
| cr26 | Pradera | Leaf | 3.9 | 13.5 | 2 | 5 | 3 | 2 | 2 | + | - |
| bitaco- | Pradera | Leaf | 2 | 95.75 | 5 | 1 | 1 | 2 | 1 | + | - |
| 20452 | Pradera | Leaf | 1.9 | - | 1 | 5 | 1 | 2 | 3 | + | - |
| 20451Ped- | Pradera | Peduncle | 2.1 | 76.88 | 4 | 1 | 1 | 2 | 3 | + | + |
| Cd8- | Quindío | Leaf | | - | 1 | 5 | 1 | 2 | 3 | + | - |
| colleyuca-27 | | Stem | 1.8 | - | 1 | 5 | 1 | 1 | 1 | - | + |

[a] Average diameter (centimeters) of colonies 72 h after planting in medium PDA + S, based on four replications.

[b] The Area Under Disease Progress Curve (AUDPC) were calculated 3, 10 and 20 days after inoculation using a scale based on symptom appearance, where 0 = total absence of lesions on stems; 1 = at least one lesion on stems; 2 = several small lesions along the stem's length; 3 = several small and medium-sized lesions along the stem's length; 4 = small, medium-sized, and large lesions along the stem's length; 5 = total plant deterioration, with lesions coalescing so that their size cannot be easily distinguished.

[c-d] The isolates were separated into five groups. The pathogenicity groups based on the disease severity rating of the one inoculated variety of soursop and diameters groups on the average of diameter of colonies growing, using ward's cluster analysis with level of confidence of 97 and 98% respectly.

[e] Three types of colonies were detected within a group of 56 *Colletotrichum* isolates. 1= Type A, very slow growing, with gray mycelium, slow and sparse sporulation, with an undulating surface, is frequently associated with lesions on very young leaves; 2= Type B, fast growing, with sparse white mycelium, and fast, abundant, and orange-colored sporulation and 3= Type C, rapid growth, with spongy, abundant, grayish-white mycelium that, on agglomerating, generates a dark compact tissue. Sporulation is moderately abundant, and the spore masses are brilliant orange and very well-defined, with drop-like forms.

[f-g] The groups were formed using genetic similarity among the isolates calculated with the Nei-Li coefficient and SAHN clustering using the unweighted paired grouped mean arithmetic average (UPGMA) method of the NTSYS-PC, version 2.02 statistical package.

[h] Specific primers for *Colletotrichum gloeosporioides.*

[i] Specific primers for *Colletotrichum acutatum.*

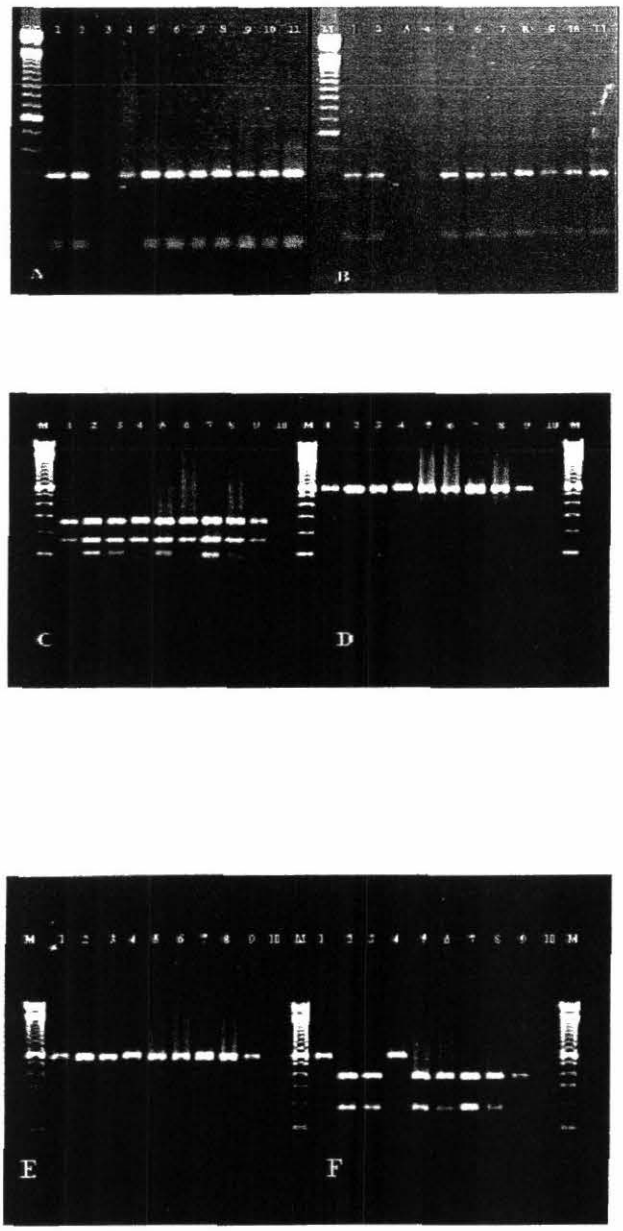


**Figure 10.** Digestion with primers ITS 1 and ITS 4 of the product amplified from 10 *Colletotrichum* isolates with the restriction enzymes (A) *Msp*1; (B) *Alu*1; (C) *Hae*III; (D) *Dra*I; (E) *Hind*III; (F) *Rsa*I. M = 100-bp marker.

A binomial matrix, where presence = 1 and absence = 0, was made with the data obtained from the digestion with restriction enzymes of the fragment amplified with the primers ITS 1 and ITS 4 and from the amplification with the specific primers. The matrix comprised 9 bands for 56 individuals that were analyzed with the statistical package NTSYS 2.02, generating a similarity dendrogram (**Figure 11**).

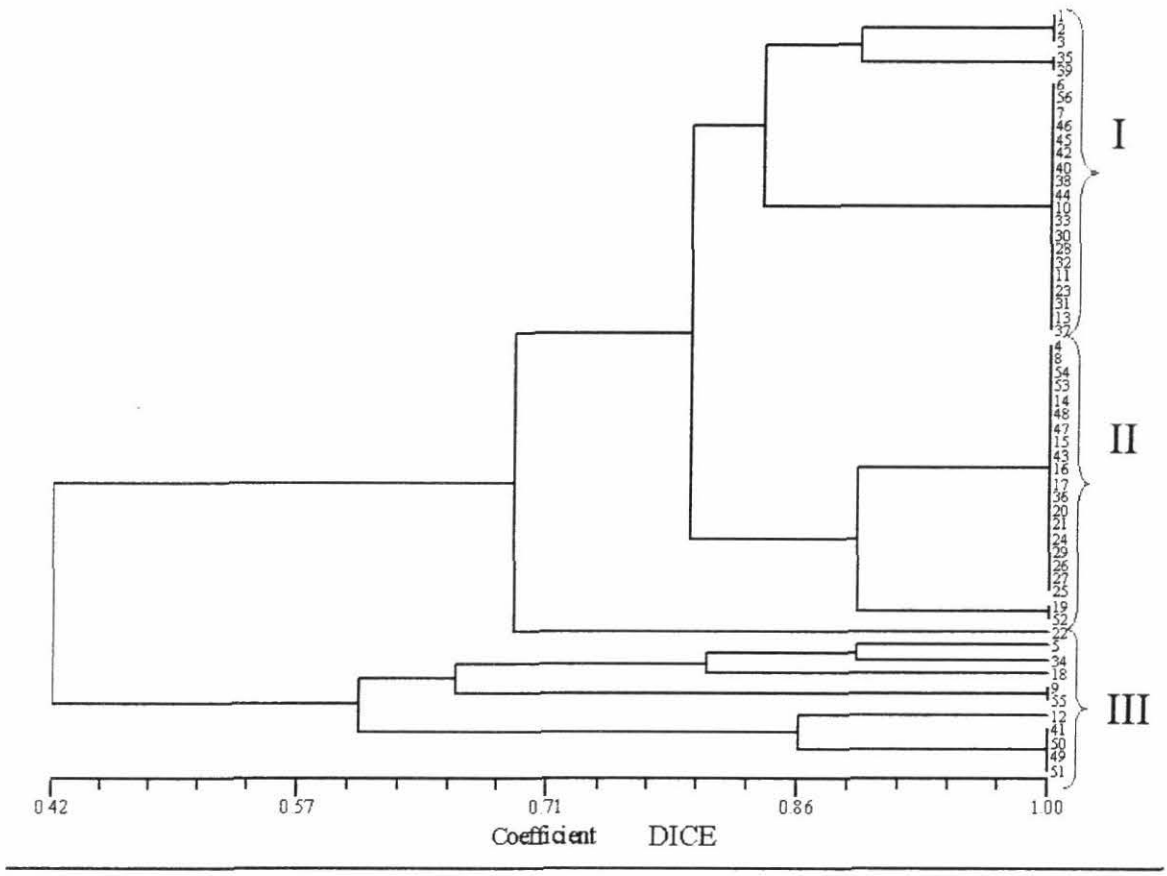


**Figure 11**. The similarity dendrogram was based on the Dice coefficient for 56 *Colletotrichum* isolates, using data obtained with the restriction enzymes *Hae*III and *Rsa*I, and amplification with the specific primers CgInt and CaInt2 in combination with primer ITS 4.

The dendrogram for restriction enzymes and specific primers showed three clusters (**Figure 11**), where the high variability of cluster III stood out, with a similarity coefficient of 0.6, to which 20% of the evaluated isolates belonged. Cluster I had a coefficient of 0.83 and contained 43% of isolates, and cluster II, a coefficient of 0.91 and 37% of evaluated isolates. In cluster III, most isolates (82%) had type A colonies, as had cluster I. Cluster II had the highest percentage (57%) of type C colonies.

The foregoing suggests that isolates with the same colony type present high genetic

variability, making necessary a molecular analysis for each morphological group (i.e., colony type), broadening the sample, and directing it toward the acquisition of larger numbers of individuals per colony types A, B, and C.

**Random amplified microsatellites.** We amplified 56 isolates with the primer CA (5' DBD A CA CA CA CA CA CA CA 3'), selected for showing polymorphism within a group of eight isolates receiving preliminary evaluation (**Figure 12**). On reading the amplified patterns, visualized by electrophoresis in a 2% agarose gel stained with ethidium bromide at 1% of a solution of 10 mg/mL (**Figure 13**). A binary matrix (where presence = 1 and absence = 0) of 30 polymorphic bands was generated.

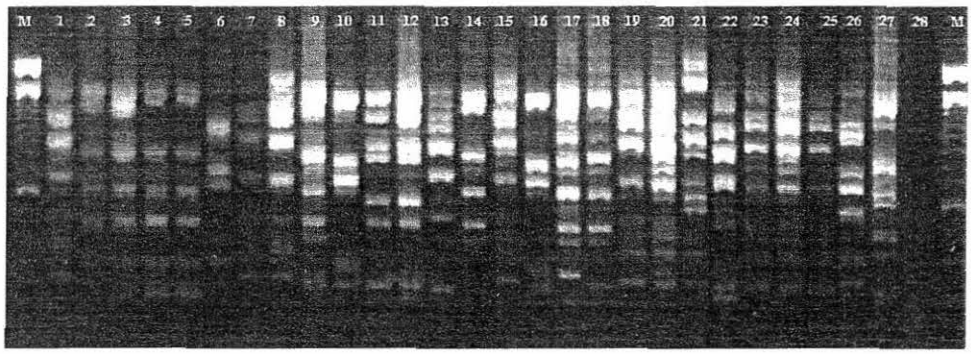


**Figure 12.** Amplification of total DNA of *Colletotrichum* isolates, using the primer CA.

The data were analyzed, using the statistical package NTSYS 2.02, to generate a similarity dendrogram based on the Dice coefficient (**Figure 13**). A cluster (A) of seven isolates, including an isolate of *C. acutatum* from cassava, was observed. Cluster B comprised 30 isolates, of which 80% presented the smallest colonies, with diameters of less than 3 cm. Cluster C had 19 isolates, of which 79% presented colonies with diameters of 3 cm or more. These findings show a relationship of the variables "pathogenic variability" and "colony diameter", with "genetic variability". The genetic variability observed with this technique is high, as at a similarity coefficient of 0.31, there is total grouping of the isolates. If the fact that sampling was restricted in terms of study area is taken into account, then we can deduce high variation exists in the pathogen's populations between different agricultural and climatic regions, with geographic isolation and high variation within groups of isolates selected previously for their morphological traits such as colony diameter. This suggests that a broader, more systematic sampling must be planned to answer the doubts arising from this preliminary study of *Colletotrichum* populations, which cause anthracnose in soursop.

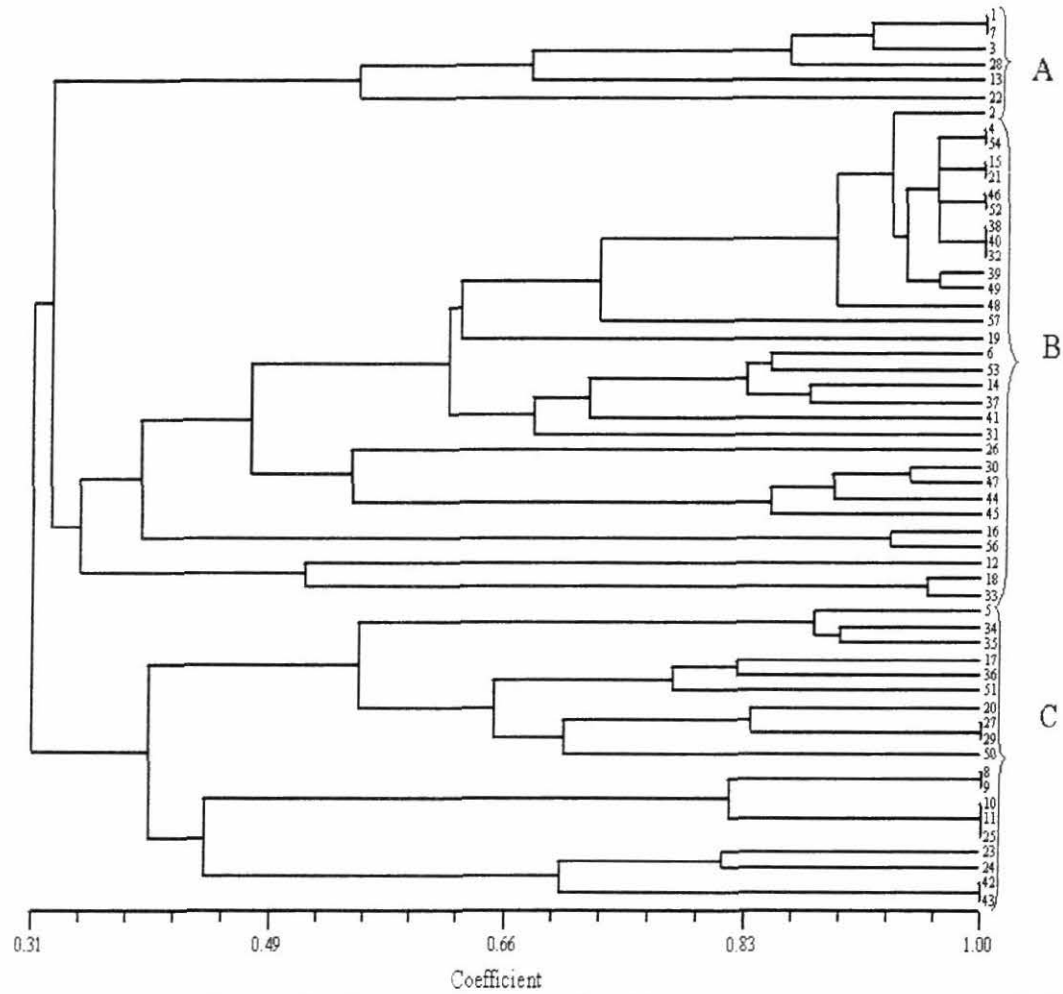


**Figure 13**. Similarity dendrogram, generated with primers RAMS, for 56 *Colletotrichum* isolates, causal agent of anthracnose in soursop.

### *4.2.3.3 Conclusions*

We detected three types of colonies within a group of 109 *Colletotrichum* isolates. Type A, very slow growing, with gray mycelium, slow and sparse sporulation, with an undulating surface, is frequen0tly associated with lesions on very young leaves. Type B is fast growing, with sparse white mycelium, and fast, abundant, and orange-colored sporulation. Type C is characterized by rapid growth, with spongy, abundant, grayish-white mycelium that, on agglomerating, generates a dark compact tissue. Sporulation is moderately abundant, and the spore masses are brilliant orange and very well-defined, with drop-like forms.

Inoculation by spraying and wounding the plant under high relative humidity (90%—98%) and at temperatures between 25° and 30°C permitted the reproduction of symptoms characterizing the disease, mainly in the stems. This confirms that *Colletotrichum* spp. are causal agents of anthracnose in soursop.

Spraying without wounding permits plant infection under the same, above-mentioned conditions, which resemble those of a rainy season for a crop in full production. The findings confirm observations demonstrating higher incidence of the disease during heavy rains and in crops with high-density planting.

An inversely proportional relationship exists between pathogenicity and colony type (correlation coefficient of -0.84), so that most isolates with slow growth had type A colonies and were highly pathogenic, whereas most fast-growing isolates had type C colonies and were moderately pathogenic. Isolates with type B colonies were not pathogenic, even though they were slow growing.

The *in vitro* methodology developed to evaluate pathogenicity made possible the reproduction of foliar symptoms characteristic of the disease on barely lignified young leaves. The isolates with type A colonies provoked symptoms of the disease in *in vitro* young leaves grown in humidity chambers, 96 h after inoculation. Isolates with colony types B and C did not provoke symptoms.

Amplifying the ITS region with the primers ITS 1 and ITS 4 did not show polymorphism among the *Colletorichum* isolates evaluated. On digesting the product amplified with restriction enzymes *Hae*III and *Rsa*I, differences were observed among the isolates, confirming that they belonged to two species. Products amplified with the specific primer CgInt and the primer ITS 4 gave a molecular size of 450 bp, corresponding to species *C. gloeosporioides.*

Of the *Colletotrichum* isolates, 9%, on amplification with the pair of specific primers CaInt2 and ITS 4, corresponded to the species *C. acutatum.*

The RAMS technique and digestion with restriction endonucleases permitted determination of genetic variability among the *Colletotrichum* isolates in Valle del Cauca.

The similarity dendrogram generated from the RAMS technique, amplifying DNA with the primer CA, showed high genetic variability and permitted the formation of three clusters that were related to pathogenicity and colony type (correlation coefficient of -0.49 and 0.65 respectly) . One cluster of 6 isolates was highly pathogenic, and was colony type A. In a second cluster, 80% of the isolates were highly pathogenic and presented the smallest colony diameters of less than 3 cm, except isolate m3612b, which was of colony type B and not pathogenic. The third cluster, formed by 79% of the isolates, was of colony type C, being moderately pathogenic and presenting diameters of 3 cm or more, except for isolates m13sra and f21, which were not pathogenic.

These results showed the variability, both phenotypic (i.e., morphological traits) and genetic (molecular traits), that exists within the causal agent of anthracnose in soursop. The results also verified that different species of the pathogen are associated with the disease.

*4.2.3.4 References*


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## 4.3 Thematic Tropical Fruit Network

This year, making use of REDECO's Internet experience to generate a flow and exchange of pertinent, updated information, the Tropical Fruits Program and REDECO plan to create a thematic network of users interested in the theme of tropical fruits. The network will facilitate identification of the demand, capture, and diffusion of specialized information, and will promote dialogue between users interested in belonging to the network.

The main objective is to capture and systematize valuable information on tropical fruits from groups of farmers and/or producers, development agencies, and agricultural ministries, according to context, returning the information to these same users to contribute to the development of their activities, via a virtual space. Specific objectives are to:

- Capture dispersed local knowledge on production systems, crop management, markets, and commercialization of different tropical fruit species, to later integrate into pilot information management tools developed at CIAT.
- Promote interactivity and interchange of knowledge and experiences among those interested in the theme of tropical fruits.

A survey in Spanish was prepared in Word format to be later sent out through the electronic list to REDECO users. The survey was published on the English and Spanish versions of the REDECO and Tropical Fruits project Web sites. The surveys were then revised and reorganized. Excel was used to store answers received, and then information was reclassified and documented. For Network administration, a directory was created in the personal address book from Outlook, and a list of addresses from the mail list server, "Mailman". The statistics package "Statistica" and SAS program were used for statistical analysis. The information was then organized in summary tables to facilitate the design of graphics in Excel.

To date, 130 replies have been received, and tabulated in Excel including basic and institutional information of those interested, the names of the five prioritized fruits according to interest, the type of information available, what information respondents required, and what they were willing to share. Of the five fruits prioritized in the 130 replies, common and scientific names and family were checked and documented. This work was complemented with the grouping of open answers according to discipline or general areas.

Countries from which interested users replied were grouped into regions:

Region 1 – Central America (Mexico, Guatemala, Honduras, El Salvador, Nicaragua, Costa Rica, and Panama)

Region 2 – Andean zone (Colombia, Ecuador, Peru, and Bolivia), and

Region 3 – Mercado Común del Sur (MERCOSUR – Argentina, Uruguay, Paraguay, and Brazil).

Region 2 had the greatest number of users interested in belonging to the Thematic Network of Tropical Fruits (69%), followed by Region 1 (24%); see Figure 1.

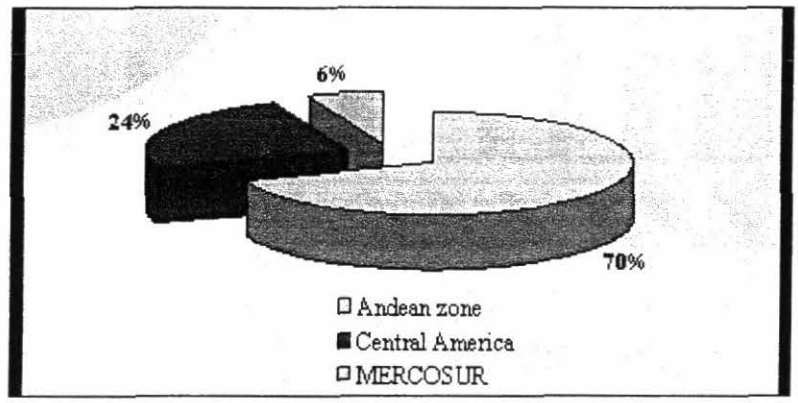



Figure 1. Percentage of replies by region of those interested in joining the Thematic Network on Tropical Fruits.

The types of institutions to which those interested belonged were academic institutions, NGOs, and national or international research institutes (Figure 2).

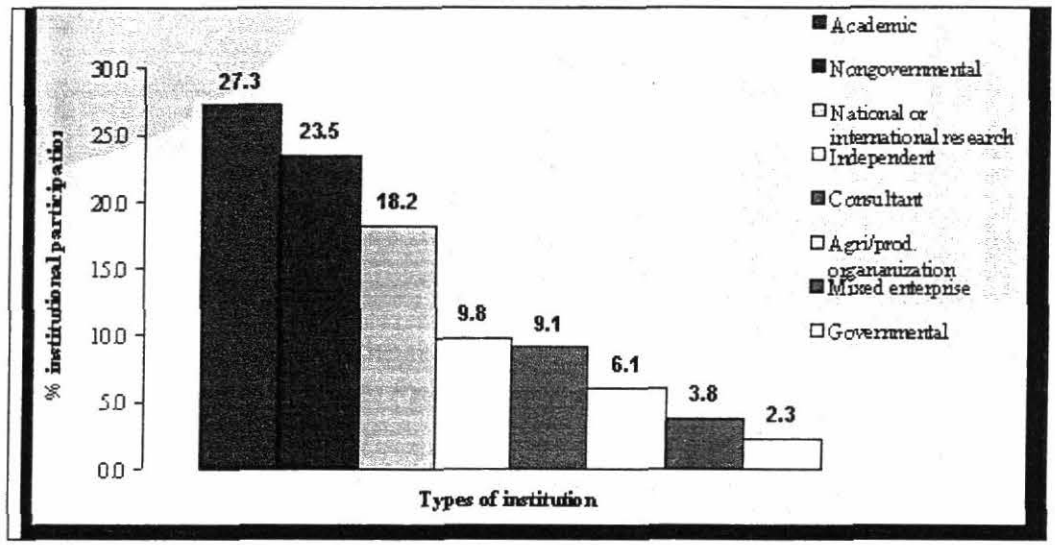



Figure 2. Type of institutions of those users interested in joining the Thematic Network on Tropical Fruits.

The most preferred fruits identified by users interested in joining the network were *Musa* spp., *Mangifera indica*, *Carica papaya*, *Citrus* spp., and *Passiflora* spp. Region 2 showed preference for *Solanum* spp, *Citrus* spp., *Passiflora* spp., *Musa* spp., *Carica papaya*, *Mangifera indica*, *Ananas comosus*, *Annona* spp., *Rubus glaucus*, and *Physalis peruviana* (in order of importance). Region 1 preferred *Citrus* spp., *Mangifera indica*, *Carica papaya*, *Persea* spp., *Musa* spp, and *Annona* spp. (in order of importance). All three regions showed some preference for *Musa* spp. (Figure 3).

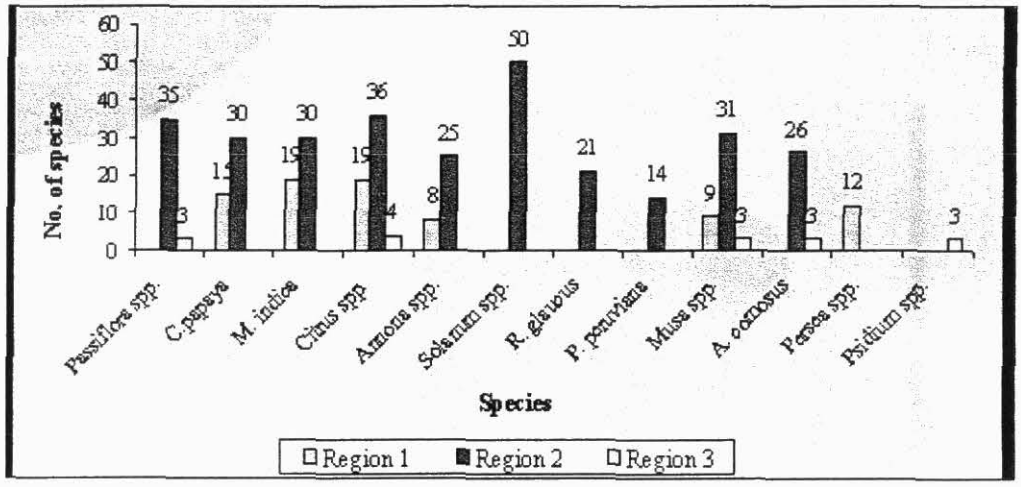



Figure 3. Fruit species prioritized by region.

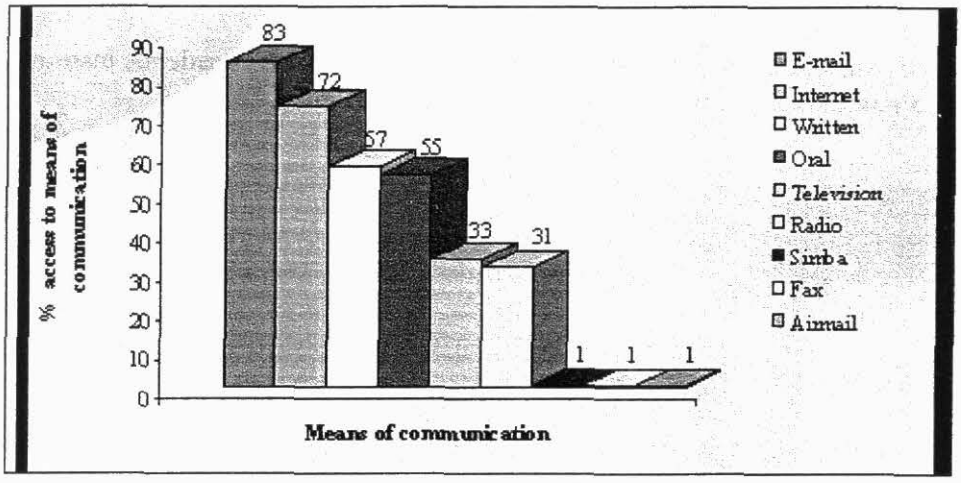



Figure 4. Percentage of access to means of communication identified by users interested in forming part of the Thematic Network on Tropical Fruits.

Figure 4 shows the channels, or means of communication, through which information reaches users.

## 4.4 Fruit flies in two departments of Colombia.

CIAT's decision to add tropical fruits to its commodity portfolio has stirred interest in analyzing the possible arthropod pest problems that might be associated with regional fruit production. The Andean region is characterized by numerous agroecosystems and an impressive diversity of crops, and this is especially true of tropical fruits. Each fruit species will have a particular pest complex associated with it. At this time, since no specific fruit species has been designated as the priority species, it was decided not to concentrate on a particular species. There are, however certain groups of pests that consist of a species

complex that can attack and damage numerous fruit species. The fruit fly complex is certainly an example of a pest that can damage numerous fruit species.

In Colombia, fruit flies are a serious problem and are found in nearly all of the fruit growing regions of the country. They are especially important and can cause considerably economic loss in the fruit export industry. In Latin America, about 20 fruit fly species have been reported causing losses calculated at about 25 million dollars per year.

The objectives of this initial study are to:

1. Establish a reference collection of fruit fly (*Anastrepha* spp) from the fruit growing regions of Valle del Cauca, Tolima and Quindío Departments of Colombia.
2. Sample and identify fruit hosts and the associated fruit fly species in the region.
3. Develop laboratory rearing methods to eventually study the biology and behavior of these species.
4. Initiate a literature search to establish a databank of present knowledge on fruit flies in the regions.

### *4.4.1 Literature Search*

Fruit flies belong to the Order; Diptera and the Family: Tephritidae. Worldwide, approximately 4000 species have been described and 400 species are reported from the Americas (Núñez; 2000). In Colombia, the most important species belong to the genera *Anastrepha*, *Toxotrypana*, and *Ceratitis*. Of the three genera, *Anastrepha* is considered to be the most important economically, owing to the considerable damage it causes on different fruit species throughout the continent (Caraballo, 2001). The origin of this genus (*Anastrepha*) is the neotropics and it consists of more than 200 described species, of which, four are considered most important economically, *Anastrepha striata* Schiner, in guayaba; *A. fraterculus* (Wiedmann) in peach, mango, plum and others; *A. obliqua* (Macquart) in mango and plum, and *A. serpentina* (Wiedmann) in níspero (persimmon), caimito (star-apple) and other sapotaceous fruits. In addition two species, *Anastrepha pickeli* and *A. manihoti*, attack cassava

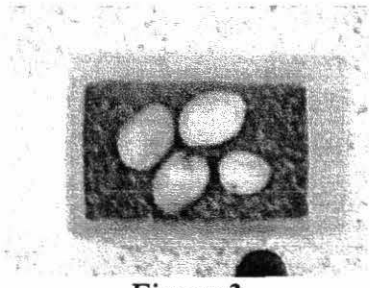


Figure 3

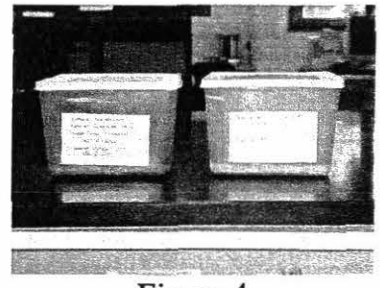


Figure 4

**Figures 3 and 4. Larval development, pupation chambers for fruit flies (*Anastrepha* spp); chambers contain humid sterile soil for pupation.**

fruits (and under certain conditions, cassava stems) but they are not considered as economically important.

Many *Anastrepha* species may be host specific; others will attack host plants within the same family. Examples of the latter include *A. grandis* attacking Cucurbitaceae, *A. oblique* attacking Anacardiaceae, *A. serpentina* on Sapotaceae, *A. striata* on Mirtaceae and *A. pallidipennis* on Passifloraceae. Generalist species such as *A. suspensa*, the Caribbean fruit fly, *A. fraterculus*, the South American fruit fly and *A. ludens*, the Mexican fruit fly attack more than 60 diverse species. These species may also have numerous wild hosts that have not yet been identified. There are 15 *Anastrepha* species recorded in Colombia, usually found between 15 to 29°C and

**Table 1. Sites sampled in the Department of Tolima, Valle del Cauca and Quindío (Colombia) for fruit fly (*Anastrepha* spp) infested fruit.**

| Department | Municipality | Locality | Host | Date |
|---|---|---|---|---|
| Tolima | Ibagué | | *M. indica* | 28 - VIII - 02 |
| Tolima | Ibagué | | *P. domestica* | 28 - VIII - 02 |
| Valle del Cauca | Cerrito | Sta. Elena | *P. guayava* | 12 - IX - 02 |
| Valle del Cauca | Bolívar | Plaza Vieja | *C. papaya* | 24 - IX - 02 |
| Valle del Cauca | Bolívar | San Fdo. | *P. guayava* | 24 - IX - 02 |
| Valle del Cauca | Bolívar | San Fdo. | *A. chirimoya* | 24 - IX - 02 |
| Valle del Cauca | Bolívar | San Fdo. | *M. indica* | 24 - IX - 02 |
| Valle del Cauca | Palmira | CIAT | *P. guayava* | 02 - X - 02 |
| Valle del Cauca | La Cumbre | | *C. maxima* | 02 - X - 02 |
| Valle del Cauca | Candelaria | Cavasa | *C. papaya* | 17 - X - 02 |
| Valle del Cauca | Candelaria | Cavasa | *P. quadrangularis* | 17 - X - 02 |
| Valle del Cauca | Candelaria | Cavasa | *C. pubescens* | 17 - X - 02 |
| Valle del Cauca | Candelaria | Cavasa | *P. guayava* | 17 - X - 02 |
| Valle del Cauca | Candelaria | Cavasa | *A. muricata* | 17 - X - 02 |
| Valle del Cauca | Candelaria | Cavasa | *A. chirimoya* | 17 - X - 02 |
| Valle del Cauca | Candelaria | Cavasa | *M. indica* | 17 - X - 02 |
| Quindío | Montenegro | Varaya | *P. doméstica* | 26 - IX - 02 |
| Quindío | Montenegro | Varaya | *M. cordata* | 26 - IX - 02 |
| Quindío | Quimbaya | El Laurel | *P. guayava* | 26 - IX - 02 |
| Quindío | Quimbaya | Querman | *M. esculenta* | 26 - IX - 02 |
| Quindío | Circasia | La Cabaña | *P. guayava* | 26 - IX - 02 |
| Quindío | Circasia | Barcelona Baja | *P. guayava* | 26 - IX - 02 |
| Quindío | Armenia | La Primavera | *M. esculenta* | 28 - IX - 02 |

from sea level to 2000 m.a.s.l. (Portilla, 1994).

Adult Tephritidos are about the size of a housefly and characterized by various colors, but predominantly yellow and translucent wings with longitudinal or transverse sports and bands. Adults live 1 to 3 months and females sexually mature in 3 to 4 days, copulating frequently (Portilla, 1994). Their biological development is influenced by humidity,

temperature, light, native vegetation, pupation and ovipositional substrate and food availability.

Eggs of *Anastrepha* spp are usually a pale white, transparent and oviposited individually. A fully developed egg is opaque and the first instar larvae are evident before hatching. The larvae are wedge-shaped with a rounded posterior. They are usually cream colored to yellow, but color can be influenced by food. There are three larval instars before pupation. Pupae are 1.4 to 1.8mm long and light straw colored to dark brown.

After copulating, females oviposit within the host fruit and the emerging larvae pass their instars feeding on the fruit pulp. The third instar larvae leave the fruit and pupates in the soil. Adults emerge within several days. The larval phase can vary from 13 to 28 days and pupae duration is 14 to 23 days. The preoviposition period is approximately 13 days; females can deposit 10 to 110 eggs per batch in fruit.

Third instar fruit fly larvae upon emerging from the fruit will pupate in the soil; pupa were removed, washed in distilled water and placed in glass gars, also containing sterile soil, where adults emerged **(Figures 5 and 6)**. Adults were maintained on water plus bee honey solution for 2 to 3 days until the complete coloration for each species was attained. Those specimens separated for identification were placed in 60% alcohol; others were mounted on entomological pins and stored in the CIAT Arthropod Reference Collection.

Basically, three morphological characters are used in the identification of fruit flies; these are the thoracic design, the wing design and the female ovipositor. Based on these parameters, the wings and ovipositors of females were mounted to facilitate identification. This was done by removing the wing of each specimen and ovipositor and placing them on a glass slide with Hoyers media. Identification was done at the ICA (Instituto Colombiano Agropecuario) Laboratorio de Sanidad Vegetal in Palmira (Valle del Cauca).

**Figure 5**

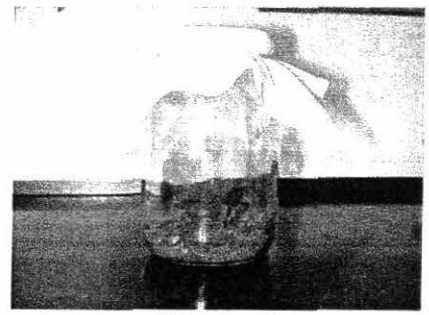


**Figure 6**

Figures 5 and 6. Fruit flies pupae collected from development chambers are washed in distilled water and placed in glass gars for adult emergence.

### *4.4.2 Results*

The fruits collected in Quindío and Valle del Cauca were mango, guava, papaya, cassava, chirimoya, plum, zapallo (calabash), sour-sop (guanábana), zapote (sapodilla), passion-flower (granadilla) and parayuela **(Table 2)**. 229 specimens were collected from these fruits and this resulted in six separate *Anastrepha* species (Table 2). The species *A. striata* was collected

from guava in several localities in Quindío and Valle del Cauca. There are several other species reported from guava from these regions including *A. fraterculus, A. oblicua* and *A. ornate.* The fact that only *A. striata* was collected from guava may have something to do with the timming of the collections, September to October 2002. This supports the need to sample fruits throughout they year in order to determine if seasonality exists for the different *Anastrepha* species and the time of fruit infestation.

**Table 2. *Anastrepha* (fruit fly) species collected from several hosts in Department of Tolima, Valle del Cauca and Quindío, Colombia (Sept. to Oct. 2002).**

| Code | Host | Department | Municipality | Identification |
|---|---|---|---|---|
| 01 | *M. indica* | Tolima | Ibagué | *Anastrepha oblicua* Macquat 7♀ 6♂ |
| 02 | *P. domestica* | Tolima | Ibagué | *Anastrepha fraterculus* Wiedmann 11♀ 10♂ |
| 03 | *P. guayava* | Valle del Cauca | Cerrito | *Anastrepha striata* Schiner 14♀ 21♂ |
| 04 | *P. guayava* | Valle del Cauca | Bolívar | *Anastrepha striata* Schiner 5♀ 7♂ |
| 05 | *P. guayava* | Valle del Cauca | Palmira | *Anastrepha striata* Schiner 136♀ 155♂ |
| 06 | *C. maxima* | Valle del Cauca | La Cumbre | *Anastrepha grandis* Trochez 2♀ 5♂ |
| 07 | *P. guayava* | Valle del Cauca | Candelaria | *Anastrepha striata* Schiner 3♀ 5♂ |
| 08 | *P. doméstica* | Quindío | Montenegro | *Anastrepha striata* Schiner Oblicua 2♀ 8♂ |
| 09 | *M. cordata* | Quindío | Montenegro | *Anastrepha nunezae* Steyscal 20♀ 10♂ |
| 10 | *P. guayava* | Quindío | Quimbaya | *Anastrepha striata* Schiner 1♀ |
| 11 | *M. esculenta* | Quindío | Quimbaya | *Anastrepha pickeli* Lima 3♀ 4♂ |
| 12 | *P. guayava* | Quindío | Circasia | *Anastrepha striata* Schiner 4♀ 7♂ |
| 13 | *P. guayava* | Quindío | Circasia | *Anastrepha striata* Schiner 5♀ 7♂ |
| 14 | *M. esculenta* | Quindío | Armenia | *Anastrepha pickeli* Lima 3♀ 4♂ |

The identifying morphological characteristics of four of the collected species are shown in figures 8 to 11.

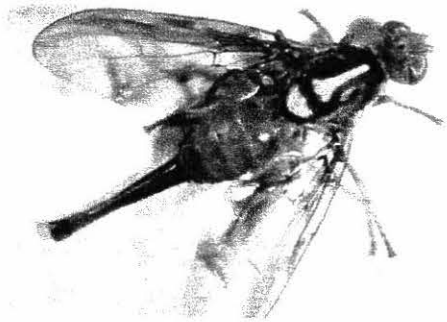
A. *Anastrepha striata*

B. Ovipositor

C. Wing pattern

**Figure 8. This species, known as the guava fruit fly, primarily attacks fruit of the Mirtaceae family but may also infest mango and sour orange (*Citrus aurantium*).**

A. *Anastrepha grandis*

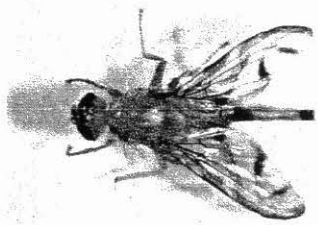


B. Ovipositor

C. Wing pattern

**Figure 9. At present, *A. grandis* is not considered of major economic importance in Colombia as it mostly attacks cucurbitaceous (i.e. watermelon). It is considered as a quarantine pest in Argentina and Uruguay and may eventually have greater importance in Colombia.**

A. *Anastrepha pickeli*



B. Ovipositor

C. Wing

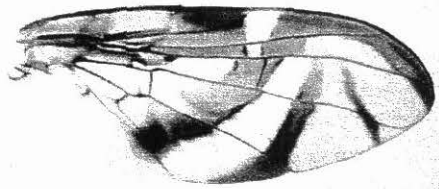
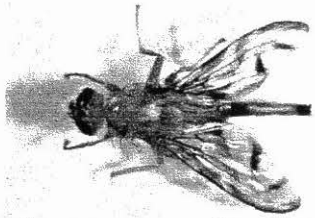
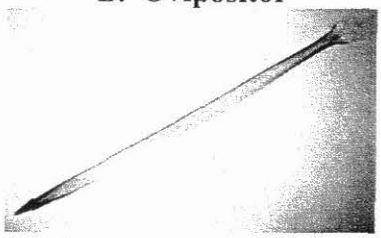


**Figure 10. This species has only been found attacking cassava fruits (and stems). When infesting cassava stems it can severe rotting due to the invasion of soft rot bacteria *Erwinia caratavora*. The latter causes a reduction in the quality of planting material (stem cuttings).**

A. *Anastrepha nunezae*



B. Ovipositor



C. Wing

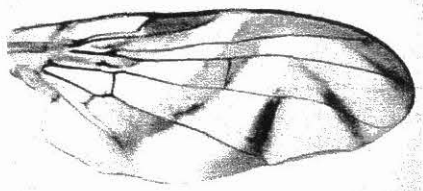


**Figure 11. *A. nunezae* was found infesting zapote (*Quararibaea cordata*) especially between 900 to 1700 m.a.s.l.**

## 4.5 Towards New Research Policies for Fruit Crop Research

The Colombian Ministry of Agriculture and Rural Development (MADR) requested assistance from CIAT in designing research policies to support tropical fruit based enterprises. Working closely with the MADR and CORPOICA we have developed guidelines for more effective use of research resources on Tropical Fruits. Although the policies are directly related to the Colombian situation, many of the recommendations are highly relevant to many other developing countries.

Fruit producers and all the actors who form part of the production chain[1] depend to a greater or lesser extent on good technology in order to be competitive, particularly when their efforts are dedicated to export markets. Good technology is necessary to improve productivity and the quality of the final product and to maintain commercial enterprises in the face of new threats. Furthermore, effective technology depends on continued research support. Thus, for any agro-business to be competitive it must be supported by research and technology development: fruits and vegetables are no exception to this rule.

Although research is sometimes seen as just another expense, there is no doubt that investing in agricultural research is highly profitable, providing economic benefits for the actors in the production chain, and social benefits in terms of lower prices and improved quality for the consumers. A meta analysis of the internal rates of return indicated that these are high in all countries studied 2. In spite of these high rates of return, research investment has been much lower in the developing countries than the developed countries in the past few decades. The research intensity3 in the agricultural sector is approximately 0.62 in the developing countries (and only 0.14% in Colombia), whilst it is about 2.6% in the developed countries4. It is probable that the low competitivity of Colombian producers is at least partially due to extremely low research intensity in agriculture over many years, and without doubt very little research has been carried out in the horticultural sector

A marked difference between the developed and the developing countries is the lack of private sector research investment in the developing countries. Recently it has been suggested that private non-profit organizations could be an interesting alternative to close the gap between private and public sector research in the developing countries. In the case of Colombia this is already occurring with a considerable proportion of the total research effort being carried out by the CENIs (National Research Centres) financed by para fiscal funds obtained by levies on specific agricultural products. The CENIs now form an integral part of the national agricultural research system. The CENIs only support those production

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[1] The production chain defined as the "Group of economic agents who participate directly in the production, transformation and movement to the markets of the same agricultural product". Duruflé, Fabre y Young. Translated to Spanish by IICA)
www.prompex.gob.pe/prompex/Inf_Sectorial/Agro/PDFMACA/PilarCoral.pdf

[2] Julian M. Alston, Connie Chan-Kang, Michele C. Marra, Philip G. Pardey, and TJ Wyatt. 2000. A Meta-Analysis of Rates of Return to Agricultural R&D: Ex Pede Herculem?. Research Report 113 IFPRI, Washington, D.C.Alston et al 1999

[3] Research intensity is defined as research expenditure as a percentage of the total gross product value of the sector involved.

[4] Philip Pardey and Nineke Bientema (2001) Slow Magic. www.ifpri.org/pubs/fps/fps36.htm

*4.5.1.2 New or Incipient Production Chains.*

The essence of the research and development in new or incipient production chains is to develop new options for the horticultural sector. Currently there is tremendous interest in developing new options for export.

**Research and Development Characteristics.** The initiative for developing new crops often comes from independent producers, however, the pioneers are often not able to appropriate a large proportion the benefits of their investment and the knowledge they have obtained. In other words they are producing a public good in the form of improved and accessible knowledge, which can be utilized freely by the rest of the population. For this reason private entrepreneurs are reluctant to develop totally new options on their own.

In agriculture and horticulture the most successful crops are normally introduced species grown far from their centre of origin. Consequently there is a high probability that the most promising new options will be species from distant lands, and local development agencies and researchers are probably not aware of their existence. In addition, agencies that are looking for new options are accustomed to looking at what are considered promising local species, or those grown by neighbors.

The research and development efforts geared to providing new options has to work simultaneously on two fronts: the research must determine what new products could be produced in a particular regions, and how those new products can be marketed.

The optimal strategy for obtaining information on potential crops or species is by visiting areas similar to the target areas in terms of ecological conditions, but which are geographically distant. Once exciting prospects have been identified, exchange of germplasm and technical know will be necessary. On the other hand marketing may be a case of opening up totally new markets or of competing in previously established markets. Marketing of export crops will also have to be closely linked with quarantine problems of both importing germplasm, and exporting fruit products with attention being paid to assessing risks.

**Implementation of the model**

- It is suggested that CCI and other agencies continue to study the potential for producting and marketing new products, and that theses agencies expand the horizons to include alternatives from Africa and Asia.
- The marketing studies should be financed by ASOHOFRUCO, ONGs, official agencies such as MADR and Proexport, and international agencies.
- Universities and other research agencies such as CORPOICA and CENICAFE should assume the responsibility of looking for new crop options particularly well adapted to specific target regions of the country. The prospecting should be based on looking for alternatives that grow well in areas that are ecologically similar to the target areas
- The MADR, Colciencias, Asohofrucol and Proexport should finance these initiatives as long term development efforts.

- The Centre of Phytosanitary Excellence should continue in its role of risk assessment broadening its scope to include a wider range of options, particularly those from other continents.

#### *4.5.1.3 Technology Export*

The possibility of exporting fruit technology, as opposed to fruits themslevles, is a novel and probably polemical idea for the developing countries. Nevertheless, there are two incontrovertible facts: the majority of important export crops are cultivated more widely outside their centre of origin, whilst at the same the basic germplasm required for their development resides principally in the centers of origin. At present Colombia imports technology in the form of improved seeds, a wide range of purchased inputs and most of the machinery for production and processing. At present there is little incentive for the country to collect and develop its germplasm if the likely beneficiaries are farmers ins other parts of the world. The question them becomes one of how to ensure that the exchange of knowledge and germplasm can be made economically attractive for those in the centre of origin who collect, and improve germplasm, and for those who use the technology for production. It appears that there may be a possibility to exploit local germplasm and biodiversity by improving it and exporting the results.

The policies required to achieve this are still being developed.