

**Citrus Canker:
Identification in the Field
and in the Laboratory**

BY: RUI PEREIRA LEITE JR

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Contact: Steve Huffstutlar, Chief of Party

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Edificio Los Tiempos Piso 7
Telf. 252096, 251655, 257827, 530278, 530149 Fax. 232773
Cochabamba, Bolivia

EXECUTIVE SUMMARY

This short-term consultancy was planned to train technical personal of IBTA\Chapare and IIA "El Vallecito" U.A.G.R.M., Santa Cruz, Bolivia, in the field and laboratory identification of citrus canker. Citrus canker has been present in the Chapare and Yapacani regions. The Chapare is located in the tropics of Bolivia and has a humid and warm tropical climate. Citrus has been grown in the region for almost 30 years. Most of the citrus orchards are small, with only a few hundred trees. The fruits are basically for the Bolivian fresh market. The Yapacani area is located on the border with the Chapare region. This is the most important area of citrus nursery in Bolivia, producing around 2 million nursery trees a year. The nursery trees produced are commercialized in almost all the different citrus producing areas of Bolivia. Citrus canker has been found in at least 40% of the nurseries of Yapacani, which comprises more than 800,000 nursery trees. Citrus canker has been introduced in the Chapare through nursery trees brought from Yapacani nurseries.

The main objectives of this consultancy were:

1. To carry out field training of technical personal of IBTA\Chapare, and IIA "El Vallecito" U.A.G.R.M., Santa Cruz, for identification and diagnosis of citrus canker.
2. To carry out laboratory training of technical personal of IBTA\Chapare, and IIA "El Vallecito" U.A.G.R.M., Santa Cruz in the preparation of culture media, inoculum processing and handling, and bacterial identification.
- 3.

Background Information

Citrus canker

Citrus canker caused by *Xanthomonas axonopodis* pv. citri (Hasse) Vauterin et al. (syn = *Xanthomonas campestris* pv. citri (Hasse) Dye)) is a serious problem for citrus production. The disease affects all above ground parts of the citrus tree causing lesions on leaves, stems, and fruits. The citrus tree may be debilitated under severe incidence of the disease due to defoliation and dieback. Fruit drop also occurs. Diseased fruits remaining in the tree do not have their internal quality affected, but they are not suitable for the fresh market.

Citrus canker lesions start as raised, creamy colored pinpoint spots. These raised lesions enlarge, becoming spongy and light brown colored. They are usually surrounded by a yellow halo, and are raised on both sides of the leaf. Usually, all the lesions on a leaf tend to be of the same size, because of the short period of susceptibility of the leaves. On fruit, the lesions are corky and crateriform and superficially similar to those on leaves. The lesions on fruit can vary in size, because the rind is susceptible for a longer time and more than one cycle of infection may occur. On stems, the lesions are also raised, corky, and creamy colored. Lesions become visible about 7 to 10 days after infection of the plant tissue. The symptoms of citrus canker can be misidentified with other diseases of citrus, such as scab, leprosis, antracnosis, etc.

Citrus canker caused by *X. axonopodis* pv. citri is the most important and widespread bacterial disease in citrus. The bacterium affects all the citrus species, such as oranges, mandarins, lemons, limes, and grapefruits, as well other rutaceous plants of closely related genera as *Fortunela* and *Poncirus*. Some citrus varieties are highly susceptible to the disease, as Mexican lime (*C. aurantifolia*) and grapefruit (*C. paradise*). Other xanthomonads may cause disease on citrus. Strain B of *Xanthomonas axonopodis* pv. aurantifolii (syn = *Xanthomonas campestris* pv. citri strain B) causes canker B on lemons (*C. limon*). This bacterium has been reported only in Argentina, Paraguay, and Uruguay. Strain C of *Xanthomonas axonopodis* pv. aurantifolii (syn = *Xanthomonas campestris* pv. citri strain C) causes canker C on Mexican lime and has been reported only in Sao Paulo state, Brazil. Bacterial spot caused by *Xanthomonas axonopodis* pv. citrumelo (syn = *Xanthomonas campestris* pv. citrumelo) is another disease caused by xanthomonads on citrus.

However, this disease has quite distinct symptoms and it has been reported only in Florida, USA.

Leaves, stems, and fruits become resistant to citrus canker as they mature. Leaf and stem infections usually occur within 42 days after beginning growth. For fruits, the most critical period for infection is within the first 90 days after petal fall. Conditions that delay maturity of the citrus tissue or favor more frequent flushes of the plant increase the chance of infection. Besides the natural infection through stomates and lenticels, the bacterium may invade the host tissue through wounds made by thorns, insects, blowing

sands or other agents. The damage caused by the leaf miner (*Phyllocnistis citrella*) particularly on leaves is very important for creating openings for bacterial infection.

The optimum temperature for citrus canker development is 28-30 C, with the minimum of 5 C and the maximum of 35 C. The presence of free water on the plant surface favor the release of bacterial cells from the lesions that can spread to infect young leaves, stems and fruits. The disease may reach epidemic proportions under wind-driven rain, with wind speed above 8 m/s. The bacterium is spread by wind and rain over short distances, i.e. within trees or neighboring trees.

Long distance spread occurs with the movement of diseased plant material, such as budwood, seedlings, or grafted nursery trees. The bacterium can be spread at long distance through diseased fruits. Orchard and nursery workers can also carry the bacterium in contaminated equipment.

Citrus in the Chapare region

Citrus is the most extensively planted crop in the Chapare, covering approximately 12,000 ha. The crop has been introduced in the region for at least 25 to 30 years ago. Most of the old groves were established with non-grafted trees of the sweet orange Criolla (*C. sinensis*), a seedy early maturing variety. Growers are still exploiting these orchards. More recently, improved citrus varieties have been introduced, such as sweet Valencia orange, Ponkan, Willowleaf, and Murcott mandarins, and Tahiti and Sutil limes. It is not difficult to find 15 years old orchards of sweet Valencia orange, and Ponkan and Willowleaf mandarins. The trees of these new varieties are usually grafted and the major rootstock used is the Cleopatra mandarin. All citrus production in the Chapare is for the fresh market in Bolivia.

The new groves have been planted with grafted nursery trees.

There are few small nurseries in the Chapare, which may provide nursery trees for the growers. However, these nurseries were established within or nearby orchards. The nurserymen usually get the budwood from their own orchard. Some nurseryman may selected the citrus trees to get better quality propagation material. The nurseries are not well managed, and the nursery trees are not uniform and they are left to grow freely without formation pruning. More recently, nursery trees have been introduced from the Yapacani area of Santa Cruz. The nursery conditions in the Yapacani area are not very different from what can be seen in the Chapare.

Bacterial Disease Diagnosis

Bacterial diseases of plants can be diagnosed based on the examination of characteristic symptoms that they produce. A typical symptom of bacterial infection is the watersoaked aspect of the lesion. This symptom can be observed by the naked eye under field or lab conditions.

In respect to citrus canker, the erupescient lesion on the citrus plant caused by infection of *X. axonopodis* pv. citri is very typical and can be usually diagnosed in the field. However, the corky symptoms at times may not be very characteristic, and the misidentification with other citrus diseases can occur.

Among the citrus diseases that are usually misidentified with citrus canker we can include leprosis, scab, and antracnosis. Furthermore, damage caused by herbicides, can be sometimes confused with citrus canker as well. Therefore, well-trained technical personal and lab facilities are necessary for the correct identification of citrus canker. In regard to this disease, the importance of citrus canker and its social, economic and political implications points to the need for a well-established procedure for its diagnosis.

Field diagnosis

The first step in the diagnosis of a bacterial disease is the identification of the symptoms present in the host tissue. The association of the symptoms observed with symptoms already described in the literature is the most appropriate way to make a correct identification of the disease based only on symptomatology. The commonest symptoms caused by bacterial diseases are canker, chlorosis, vascular discoloration, watersoaking, wilting, scab, rot and tumour.

Bacteria exudation test

The presence of a large number of bacterial cells in the infected plant tissue and the lack of other pathogens suggest that a bacterium may be the causal agent of the disease. In the case of vascular wilt, bacterial exudation can be observed by the naked eye by placing a piece of the disease tissue in water. The formation of a milky stream coming out from the plant tissue indicates that the problem is caused by a bacterial infection.

For leaf spots, the bacterial stream can be also observed by light microscopy examination of the diseased tissue. Slices of the diseased tissue are placed in a drop of water on a glass slide. This preparation is covered with a cover slip and then observed under microscope.

Gram stain

The Gram stain is a basic step to differentiate two major groups of bacteria: the Gram-positive and the Gram-negative bacteria. Under a protocol of staining and destaining, the Gram-positive bacteria retain a blue stain (crystal violet) while the Gram-negative bacteria do not retain this stain and produce red colored bacterial cells. This test is carried out under lab conditions and requires light microscopy examination of the bacterial cells. Most of the plant pathogenic bacteria, also including the Xanthomonads, are Gram-negative.

The Gram stain is an important step in the bacterial identification because it also provides information in regard to the shape and size of the bacterial cells.

Bacterial isolation and storage

Most plant pathogenic bacteria can be cultivated in pure culture on artificial media. The isolation of the bacteria from the host tissue requires several steps to eliminate contaminant microorganisms and to obtain a pure culture of the bacterial agent. Appropriate disinfection procedures and media should be used to successfully isolate the plant pathogenic bacteria.

Once isolated in pure culture, plant pathogenic bacteria can be stored for further characterization and identification. For short-term storage, the bacterial strain can be stored in appropriate culture media in test tub renewed by monthly transfer. For long term storage, the bacterial strain can be stored in water, lyophilized, etc..

Bacterial Inoculation or Pathogenicity test

The final step in the identification of a bacterial plant pathogen is the pathogenicity test. In this step, the bacterial strain is put back into the host plant to check if it is pathogenic and produces symptoms identical to the ones observed in the original diseased plant sample.

To carry out the pathogenicity test, healthy plants of a susceptible host must be kept under protected conditions, usually in a greenhouse. These plants are then inoculated with a suspension of the bacterial strain. After an incubation period of few days, the symptoms should be visible to the naked eye.

Plant Pathology Laboratory at IBTA/Chapare

A Plant Pathology Laboratory facility exists at the E.E. La Jota/IBTA located in Chimoré. The laboratory is run by Mr. José Camacho and Mrs. Carmen Calderon, technical personal of IBTA/Chapare. The laboratory has basic equipment (i.e. autoclave, incubator, refrigerator, microscope, stereoscope, balance, hood, etc..) and lab supplies (i.e. glasses and reagents) to carry out bacterial diagnoses and other plant pathology work. However, some of the equipment are old and there is a shortage of lab supplies. Therefore, there is a need for replacement of some equipment and to provide appropriate lab supplies to carry out properly routine bacterial diagnoses. Furthermore, the E.E. La Jota/Chapare does not have appropriate greenhouse facilities. Greenhouse facilities would be necessary to carry out pathogenicity test of bacterial isolates for final identification of the citrus canker pathogen.

Training of IBTA/Chapare and IIA Vallecito Technical Personal for Citrus Canker Diagnosis

Field training

During this short-term consultancy, technical personal of IBTA/Chapare, UNAGRODI, SEDAG, and IIA Vallecito were trained for field identification of citrus canker. This training consisted basically of the identification of citrus canker through visual examination of citrus plants during visits to citrus orchards and nurseries naturally infested with the disease in the Chapare and Yapacani areas. Citrus orchards of different ages and varieties were visited in the Curichal, Senda Tres, Tamborada 3, San Carlos, Ivirgazama and Bolivar areas of Chapare, and a nursery in Yapacani, Santa Cruz. An important aspect of this training was the identification of citrus canker at different stages of symptom development. Initial symptoms of the disease are usually raised pinpoint lesions on the leaf surface. In contrast, the corky aspect of the lesions usually characterizes older symptoms, with presence of an oily border, and a yellow halo surrounding the canker lesion. Sometimes, these typical citrus canker symptoms are not so evident and a careful examination is necessary to make a correct diagnosis of the problem. Further, the presence of the leaf miner (*Phyllocnistis citrella*) and its association with citrus canker poses another problem for the correct diagnosis of citrus canker.

The technical personnel were exposed to several different aspects of the citrus canker symptoms and how to identify correctly the disease under field conditions. When the diagnosis was not possible in the field, the recommended procedure was to send the sample to the lab for identification.

The differentiation of citrus canker from other citrus diseases and pest problems was also an important point in this field training. During the visits to the citrus orchards and nurseries, the technical personal was exposed to different problems usually misidentified as citrus canker, such as leprosis, citrus scab, antracnosis, and herbicide damage. The careful comparison of the symptoms caused by different agents and those caused by the citrus canker bacterium allowed the correct identification of the disease.

Laboratory training

Laboratory training on the preparation of culture media, inoculum processing and handling, and bacterial identification was provided to the IBTA/Chapare (Mr. Jose Camacho and Mrs. Carmen Calderon) IIA Vallecito (Mr. Carlos Rivadeneira Michel and Mss. Jaqueline Galvan) technical personal.

Laboratory training included microscopic examination of bacterial exudation from diseased plant tissue.

Samples of citrus canker collected during the field trip were used for the lab practice of bacterial exudation. Slices were cut from the diseased tissue and placed in a drop of water on a glass slide. This preparation was covered with a cover slip and then observed under microscope for bacterial exudation. In contrast, diseased citrus samples not caused by bacteria, i.e. scab, antracnosis, were also handled in the same way and examined under microscope. These plant materials did not show any evidence of bacterial exudation.

Laboratory training was also provided in the preparation of culture media for bacterial isolation, purification and storage. Different culture media were prepared in the

laboratory. For instance, nutrient agar medium for first isolation and purification of bacteria was prepared in Petri plates while for storage YDC medium was prepared in test tubes. Further, reagents to be used in the Gram stain technique were also prepared.

For the isolation of the bacterium from the host tissue, a protocol was followed to assure the elimination of contaminant microorganisms and to obtain a pure culture of the citrus canker bacterial agent. Appropriate disinfection procedures and media were used to successfully isolate the plant pathogenic bacterium. Cultures of the citrus canker bacterium were established and appropriately stored in water and test tubes. All these steps were practiced by the technical personal of the Plant Pathology Laboratory.

The Gram stain technique, a basic step in the identification of plant pathogenic bacteria, was also carried out successfully by the technical personal. Under the protocol used, the citrus canker bacterium was correctly identified as a Gram negative bacterium. The Gram stain was also important to observe the shape and size of the cells of the citrus canker bacterium and to differentiate from saprophyte bacteria.

Update on Citrus Canker in the Chapare

Citrus canker has been reported in the Chapare of Cochabamba and also in the Yapacani area of Santa Cruz de la Sierra. Although the disease seemed to be restricted to infected nursery trees recently introduced in the Chapare, new observations revealed that 3 to 4 years old citrus orchards planted in the area have been infected with citrus canker. Based on the symptoms observed, there are evidences that contaminated nursery plants were also the source of the disease in those older orchards. Secondary spread of the pathogen tree to tree is now occurring in the Chapare. New citrus canker infections are very clear and an epidemic status of the disease may occur soon if contaminated measures are not effectively adopted to suppress the spread of the disease. Furthermore, the Chapare has very favorable environmental conditions for the development of the disease, with high temperature and almost daily rains. The leaf miner, which causes severe damages to citrus, and favors the development of citrus canker, is also doing its work in the Chapare. In several cases, citrus canker symptoms were associated with leaf miner damage to the citrus leaves.

The source of citrus canker in Bolivia has been the nurseries in the Yapacani area of Santa Cruz de la Sierra. In this area, more than 2 million nursery trees are produced yearly. These nursery trees are commercialized in almost all the citrus growing areas of Bolivia. Based on surveys carried out in the Yapacani area, citrus canker is present in more than 40% of the nurseries, which are responsible for the production of more than 800,000 nursery trees. Despite some efforts of the officials of the Department of Agriculture of the municipality of Yapacani, no effective measures have been taken in the field to prevent the dissemination of citrus canker to new areas through contaminated nursery trees. During the visit to a nursery in Yapacani on May 8, contaminated nursery trees were ready for commercialization. Furthermore, the disease is also present in orchards and nurseries of other municipalities of the Ichilo province.

Concluding remarks

A field and laboratory training was provided for the IBTA/Chapare, SEDAG, UNAGRODE and IIA Vallecito technical personal in the identification of citrus canker during this short-term consultancy. Technical personnel were able to identified correctly citrus canker under field conditions, and also to differentiate it from other citrus diseases. The laboratory of Plant Pathology at the E.E. La Jota/Chapare is also capable to carry out lab identification of citrus canker. Some improvement in the laboratory will be necessary to assure better conditions for working more safely with citrus canker.

Despite some isolated efforts, no effective measures were adopted to contain the spread of citrus canker in Bolivia in the 5 months after the first consultancy. The disease is still spreading in the field, diseased citrus plants were not eliminated and secondary spread of citrus canker is now underway in the Chapare. New introductions of the disease are occurring through contaminated nursery trees. Contaminated nursery trees are still being produced in the Yapacani and have been commercialized throughtout Bolivia.

Based on these considerations, citrus canker is likely to became endemic in Bolivia very soon. The environmental conditions and the cultural practices adopted by the citrus growers are favorable for the permanent establishment of citrus canker. The presence of citrus canker in the citrus orchards will certainly require changes in the management practices adopted by the growers to successfully grow citrus under this new situation.

May 10, 2000

Rui Pereira Leite Jr.

APPENDIX A
Program of the Short-Term Consulting

05/02	Arrival in Cochabamba, Bolivia
05/03 Morning	Travel to Chapare by car
Afternoon	Meeting with technical personal of IBTA/Chapare at E.E. La Jota, Chimoré Visit to the Plant Pathology Laboratory
05/04 Morning	Lab-work: Preparation of reagents and bacterial media
Afternoon	Visit to citrus orchard in the Chimoré area to carry out training in the field identification of citrus canker and to collect diseased samples for lab work
05/05 Morning	Lab-work: Exam of disease samples by light microscopy (bacterial exsudation) and bacterial isolation practices
Afternoon	Visit to orchards in the Tamborada 3 sector with MIP and UNAGRODI technical personal for identification of citrus canker based on symptoms and differentiation of other citrus diseases Sampling of diseased plant material for lab-work
05/06 Morning	Lab-work: Media preparation, diseased samples examination by light microscopy (bacterial exsudation and Gram stain), isolation of the citrus bacterium in pure culture
Afternoon	Field trip to San Carlos area with MIP and IIA Vallecito technical personal. Field training in the identification of citrus canker and differentiation of other citrus problems (herbicide damage, etc.) Sampling of citrus canker material for lab-work
05/07	Lab-work: examination of isolation of citrus canker bacterium, light microscopy work for identification of bacteria
05/08	Trip to Yapacani, Santa Cruz de la Sierra, with IBTA/Chapare, MIP, IIA Vallecito and SEDAG technical personal Meeting with technical personal of Yapacani and citrus growers and nurserymen

05/09 Morning	Field trip to Ivirgarsama with IBTA/Chapare and MIP technical personal for identification of citrus canker based on symptoms and differentiation from other citrus diseases
Afternoon	Lab-work: examination of isolations of citrus canker bacterium, pure culture technique, transfer and preservation of bacteria
05/10 Morning	Conclusion of lab-work Meeting with technical personal of IBTA/Chapare and IIA Vallecito Report preparation
Afternoon	Trip to Cochabamba by car
05/11	Departure to Brazil

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