Food and Agriculture Organization of the United Nations

Webinar:
"Adressing the Global Knowledge Gap on Diagnostic Tools, Protocols and their Applications for Fusarium wilt of Musa Tropical Race 4 (TR4)"

July 20, 2021

Moderator: Mr. Nelson Laville, *Sustainable Development Specialist, World Banana Forum (WBF) Secretariat, FAO*

Opening speeches:
Victor Prada, *Secretary General of the World Banana Forum (WBF), Food and Agriculture Organization of the United Nations*
Raixa Llauger, *Agricultural Officer (Tropical Fruits), Food and Agriculture Organization of the United Nations*

Panelists:
Dr James Dale, *Banana Research Program Leader, Center for Tropical Crops and Biocommodities, Queensland University of Technology*
Dr. Yolande Chilin-Charles, *Phytopathologist - Epidemiological Engineer, CIRAD, Montpellier Institute of Plant Health / Guadeloupe*
Dr. Gert Kema, *Chair at the Laboratory of Plant Pathology, Wageningen University and Research*
Dr. Diane Mostert, *Technical Director, Department of Plant Pathology, Stellenbosch University*
Dr. Miguel Dita, *Senior Scientist - Plant Health for Sustainable Banana Production, Bioversity Alliance and CIAT*
Dr. Peng Jun, *Key Laboratory for Integrated Pest Management in Tropical Crops, Chinese Academy of Tropical Agricultural Sciences*

Summary:

The World Banana Forum (WBF) is a multi-stakeholder platform that brings together all key constituencies of the banana sector, including governments, producers, exporters, importers, retailers, unions, and civil society organizations. During the process of developing the global proposal in TR4 and other consultations with stakeholders, one of the deficiencies identified was the knowledge gap on diagnostics, options, tools and access to reference laboratories. This webinar aimed members of the Global Network on TR4, the National Plant Protection Organizations (NPPOs) of the banana producing countries, researchers, laboratory technicians and producers. The event aimed to provide clarity on various issues and challenges faced during the diagnosis of TR4. A total of 400 people participated in the event.

Opening words:

Mr. Victor Prada opened the session by welcoming all the panelists and participants of the event. Mr Prada mentioned that Fusarium Wilt Tropical Race 4 (TR4) ranks high on the World Banana Forum (WBF) agenda and is likely to continue to occupy it for years to come. He commended the efforts made by the WBF and FAO in establishing the Global Network on TR4 and in developing a global proposal on the disease. He then stated that FAO is mobilizing resources to support the response to the outbreak in Peru.

Then, Mr. Prada briefly presented the activities of the Global Network on TR4, highlighting the essential participation of public and private sector actors for the implementation of integrated solutions, prevention activities, alternatives and mitigation strategies to contain the spread of the disease. Mr Prada highlighted the role of the TR4 Global Network as a neutral platform for knowledge exchange, awareness raising regarding the threat, development and distribution of capacity building materials and coordination of actions in prevention and control, creating the basis for inclusive and open collaboration —around the world—that benefits both users in the field, NPPOs technicians and decision makers.

The WBF Secretary briefed the audience on the development of a global proposal to combat the disease based on FAO's experiences gained during the implementation of technical cooperation programmes around the world. The global proposal of the TR4 Global Network was developed after extensive consultation with relevant entities and received comments from FAO technical units. He concluded by mentioning that the global proposal should be used as an example or template to be adapted to national and regional contexts and emphasized that this is precisely what FAO is doing for the Andean region.

Mrs. Raixa Llauger began her speech by thanking everyone present and the organizers of the event. She briefly presented the work carried out by FAO in Latin America and the Caribbean in the last year regarding the emergency posed by Fusarium wilt TR4. She highlighted the importance of the diagnosis of TR4 as one of the Musaceae diseases that causes the greatest socioeconomic impacts around the world. Likewise, she mentioned the relevance of advancing with prevention programmes, surveillance and management strategies for the banana sector as a whole, including small producers. Ms. Llauger highlighted the importance of reinforcing and strengthening the capacities of the countries to guarantee an adequate preparation and response to the TR4, mitigating the risks and the economic impact derived from this important pest.

The FAO's agricultural officer provided an overview of the status of the disease in the Latin American region and mentioned the technical cooperation programs developed by the regional office of FAO, including the response to the new outbreak and the formulation of a new joint project for the Andean countries. In addition, she presented other activities, such as training workshops, drills, high-level awareness meetings, promotion of government alliances with the private sector, and development of audiovisual training materials for producers and officials. She then highlighted the technical exchanges — from theory to action — carried out with the Colombian Agricultural Institute (ICA), the International Regional Organization for Agricultural Health (OIRSA) and National Plant Protection Organizations (ONPFs),

Ms. Llauger provided a summary of the actions taken in response to the recent outbreak in Peru, highlighting the technical exchanges and the acquisition of biosafety kits and disinfectants for the country. She also mentioned the development of an action plan and a joint project proposal for the region. She then informed the audience about meetings held with public and private sector actors to support the rapid response and to understand the current situation and identify intervention priorities in each of the countries of the Andean region. Ms. Llauger then concluded her speech by welcoming all panelists and participants of the event.

Plenary:

Dr James Dale presented the case study regarding the work on TR4 diagnosis conducted in Australia over a three-year period at Queensland University of Technology (QUT). The work is part of a larger project focused on developing resistant Musaceae cultivars. The experimental field is located in the Darwin area. Dr Dale explained that TR4 has been present in the experimental area for at least twenty years and can therefore be characterized as endemic in the soil. Researchers have documented the complete history of each plant present in the field trial. In the experimental area, the polymerase chain reaction (PCR) is the main diagnostic technique used, and all infections are confirmed by PCR and sequencing.

Dr Dale presented details of the experimental field including methodology and experimental designs. The planting began in March 2018, where four (4) genetically modified lines and two (2) controls (Grand Nain and William) were established in a randomized block design. The trial collected agronomic data, yield, and disease resistance information.

The monitoring of the experimental plots in 2019 indicated that no one of the genetically modified plants showed symptoms, while the controls showed yellowing of the leaves and necrosis of the pseudostem. All the collected samples were diagnosed by molecular techniques. The diagnosis was based on the SIX genes (secreted by the xylem), identified as pathogenicity chromosomes in *Fusarium oxysporum* fs. *cubense*. This genetic marker is new and very useful. The SIX genes have different sequences that can be identified in Tropical Race 4 and Subtropical Race 4 (STR4), SIX1 has three identifiable sequences in TR4, SIX13 has an identifiable sequence in TR4, SIX8 two identifiable sequences of the disease and SIX6 presents an identifiable sequence in TR4,

Dr Dale stated that those genes and sequences are diverse enough to be used in confirmatory diagnoses of Foc TR4. Therefore, based on the SIX genes, the QUT has designed its own sets of primers for diagnosis. QUT has a total of six sequenced SIX genes

- Foc TR4 has 3 SIX1 genes
- Foc STR4 has 1 SIX1 gene
- Foc Race 1 has 2 SIX1 genes
- All six SIX1 gene sequences are unique

After the observation of the first symptoms in the experimental field of QUT, the plant is cut and there is a comparison of the symptoms from the base of the pseudostem to the peduncle and the fruits. In their diagnostic results, infected plants presented positive results in PCR for samples collected at the base and pseudostem, but negative in the peduncle and fruits. Dr Dale then emphasized that TR4 was never detected in peduncles or fruits, ensuring that banana fruits are not capable of spreading the disease.

Dr. Dale concluded his speech by highlighting important messages from the QUT experience:

- To take samples, it is essential to euthanize the plant being sampled — because vascular discoloration symptoms, especially in plants with early symptoms, are unevenly distributed throughout the stem.
- It is highly desirable and essential that the PCR products be sequenced for the verification of "new incursions".
- Foc TR4 has not been identified in banana peduncle or fruits, even in cases with advanced TR4 infections.

Dr. Yolande Chilin- Charles began her presentation by describing the methodology proposed by CIRAD for the TR4 Diagnosis in French overseas territories and informed the audience that the methodology has already been validated by the plant protection services of the French Ministry of Agriculture and it has four common steps to all diagnostic protocols.

The CIRAD phytopathologist warned the participants about the importance of sample collection activities in the field. Sample collections must be accurate and efficient because even if the diagnostic tools are correct, if the sample is not collected properly, the diagnosis can be incorrect.

The diagnostic procedure in the French territories is made up of four main actors who act in monitoring the disease. They are: (i) producers, (ii) technicians, (iii) plant protection services, and (iv) partners such as Fredon and the IT2 Technical Center. All of them carry out visual inspection of the plants, take photographs and send them to the competent authorities in case of suspicion of TR4.

CIRAD is currently developing an Android-based mobile application to facilitate the collection of disease data at the field level. It is expected that the software improve the response time regarding sample collection when and where it is needed. Producers and technicians will be able to use the information available in the software database to support the identification of external symptoms. Currently, in the event of a possible outbreak, the affected area is identified and the Plant Protection Services are informed. The collection of the samples is then carried out by a delegate assigned by the Plant Protection Services. For French overseas territories, the diagnosis is made by the French Food Agency.

CIRAD's diagnostic method consists in the following steps:

- Step 1: Isolation of the fungus and morphological identification
- Step 2: Analysis of plant samples using the real-time PCR protocol developed by Aguayo and Chillin-Charles in 2017
- Step 3: Establishment of pathogenicity tests using Koch's postulate. For this test, it is used a solid inoculum (*Miletus infused with a suspension of conidiospores*)
- Step 4: Characterization by vegetative compatibility groups (VCGs) such as VGC 1213/16

Dr. Chilin-Charles mentioned that CIRAD is exploring a novel method for early diagnosis in the field, the *Diagnofus* based on the LAMP protocol, and that it will be proposed for official adoption later this year. To conclude, Dr. Chilin-Charles highlighted some key points of her presentation:

- Monitoring should be a participatory exercise which involve producers;
- The software should improve data capture and response and support the readiness of competent authorities
- Sample collection can be made more secure using Sigma FTA cards. These cards will minimize the risk of accidental spread;
- The diagnostic process for TR4 should include molecular testing and pathogenicity testing.

Dr. Diane Mostert provided an overview of the *Fusarium oxysporum* fs *cubense* TR4 characterization process at Stellenbosch University with a specific focus on new outbreaks. She stated that the laboratory analysis conducted in the university usually begins with dried infected plant material.

The methodology used by the university is diverse and based on the urgency attributed to the samples received. Dr Mostert explained that there is the possibility of using a protocol to extract DNA directly from plant material and perform a quantitative real-time polymerase chain reaction (qPCR).

Using this method the results can be available one day after receiving the samples. However, Dr. Mostert cautioned that while this technique allows quick and accurate results, it is quite expensive and requires specialized personnel. Furthermore, there is a risk of false positives when detecting the disease directly from material in the environment, since qPCR is performed using a single gene as a target. The researcher mentioned that if the samples are not collected correctly, false negative results could be obtained.

In the event of a new incursion, several conventional PCRs are used to confirm that the Foc TR4 is correctly identified. Ms Mostert noted that the shipment of infected material can be subject to regulatory scrutiny and may result in the loss of the sample. Therefore, in countries with DNA extraction capabilities, technical staff can perform their own isolation and purification and send the DNA to Stellenbosch. This method is highly recommended as it requires less scrutiny by the authorities due to the lower quarantine risk.

As part of Stellenbosch University standard operating procedures, all molecular tests are confirmed by VCGs testing — due to previously identified differences in virulence and genetic variation observed in phylogenetic evaluations. Among the disadvantages of VCG analysis is the time required and the need for live fungal material, which can present a quarantine risk.

The final stage includes pathogenicity tests using Koch's postulate. According to Dr. Mostert, this is the most reliable way to verify the virulence of the pathogen.

Dr. Mostert stressed that extreme caution should be taken before finalizing findings and drawing conclusions. The level of confidence must be ensured by verification using as many diagnostic techniques available. No molecular marker is reliable to be used alone, due to the risk of presenting false positives.

Dr. Mostert concluded by highlighting important messages:

- There are qPCR assays available that are specific to VCG 1213/16 (Foc TR4) with applications in water, soil and plant material samples.
- When designing molecular markers, it must be taken into account that the larger the number of genes covered, the more accurate the diagnosis.
- When strengthening the diagnostic capacity, the situation, the budget and the skills (human capacity) available must be taking into consideration to decide the most appropriate tool for the situation.

Dr. Gert Kema began his presentation by congratulating the World Banana Forum for its more than ten years of existence and remembered his participation in the Forum since its inception. In his presentation, Dr. Kema provided an overview of the symptoms of Fusarium TR4 and the sample collection process. He presented to the audience the global spread of the disease over time, highlighting the recent incursions in Latin America. Dr. Kema emphasized the importance of having quick and accurate diagnoses when a new incursion occurs.

Dr Kema presented the diagnostic techniques carried out at Wageningen University and Research (WUR) emphasizing the various methods conducted, including pathogenicity tests following Koch's postulate. The researcher mentioned that the University of Wageningen has recently abandoned the testing of VCGs because all the strains collected by the laboratory are sequenced, not needing this type of analysis.

In the context of the speed and precision of the diagnosis, he gave the rationale for the commercialization of diagnostic kits, emphasizing its advantages as being accessible, standardized and with a reduced margin of error.

He clarified that the most widely used molecular tool is PCR, using different parts of the TR4 genome. According to the researcher, scientific articles have been published to support other target areas of the genome fordiagnosis of TR4 and he believes that the more they are used, the more reliable the diagnosis will be.

However, Dr. Kema cautioned that there is no single diagnostic technique that will last forever. As pathogen populations change, diagnostic processes will need to be updated. LAMP is an example where it uses a different

section of the genome. He predicted that lateral flow devices (LFD) may be available soon to support rapid TR4 testing at the field or laboratory level. Dr. Kema briefly presented WUR's activities related to fungal phylogeography and genetic sequencing analysis used to predict and infer the possible origin of new incursions.

Dr Kema compared the PCR, LAMP and LFD diagnostic methods with respect to the substrate used for the analysis (plant material, water and soil samples), the investment and training required and the performance of each method, highlighting their advantages and limitations. Dr Kema concluded his intervention by acknowledging the support of donors and collaborators for the work carried out by WUR and presenting the following messages:

- The use of different parts of the TR4 genome for diagnosis is recommended.
- Diagnostic techniques are not fixed and evolve over time.
- With the evolution of techniques, it is very likely to have a rapid test for TR4 similar to the one developed for COVID-19.

Dr. Miguel Dita titled his presentation “From field diagnosis to official confirmation”. In his speech, he regretted that sometimes the link between the diagnosis of TR4 and the legal framework in the countries are not clearly understood. He them provided an overview of the diagnostic methods applied for TR4, from 2008 to 2020 highlighting the differences regarding the detection of the disease by research institutions and the steps required for the official declaration of the presence of TR4 by the official authorities (National Plant Protection Organizations). According to Dr. Dita, the official declaration depends on strict and precise diagnostic protocols and may incur in social, economic and political implications for the country.

Diagnosis is essentially a process; it is not a tool. According to Dr. Dita, it is essential to understand how technical tools are incorporated into the flow of the diagnostic process, especially within the legal framework of a country where there is a new incursion. As part of the diagnostic process, recognition of symptoms in the field is essential. There are multiple factors and complexities that must be taken into account during the monitoring process in the filed, the ability to identify TR4 when it occurs in combination with other diseases and / or abiotic stresses is crucial for field technicians.

According to the scientist, there are several protocols for sampling. The Contingency Plan developed by OIRSA speaks very clearly on the subject. Sample collection should be conducted by NPPO staff to the greatest extent possible. Sample processing capacity may seem trivial, but it is a very important link between microbiology lab work and molecular biology lab work. The ability to perform a correct isolation and purification is essential to advance in molecular diagnosis.

In the context of the NPPOs, there should be a differentiation between routine diagnosis and emergency diagnosis as in the case of a pest of quarantine importance. A specific set of skills and protocols are required to provide confirmatory results.

In 2010, the PCR technique for TR4 was published, which offers faster diagnostic results, but this does not mean that the previous technique used was not valid. Past techniques serve as building blocks for future diagnostic protocols. Referring to what Dr Kema mentioned, Dr Dita mentioned that no diagnostic technique is eternal. According to the researcher, diagnostic methods will be improved as technology evolves and new science emerges. For example, the article published in 2014 speaks of the SIX8 gene as a new marker for the molecular identification of TR4 in addition to the use of the SIX1 gene mentioned by Dr Dale and used in Australia.

The NPPO of any country is ultimately responsible for any decision regarding the declaration of the presence of a new incursion, either by VCG or VCG plus PCR and / or sequencing. These decisions are made with caution because any statement carries social, economic and political implications. Thorough analysis is required before making such statements. However, there are actions that must be taken, even before a statement is delivered. As in the case of Peru, where detection, diagnosis and containment of the disease were carried out.

Dr. Dita summarized his presentation by providing some important take-home messages:

- Science and technology have evolved to detect TR4 in plant tissue, water, and soil; however, soil and water detections should not be used to support official declarations regarding a TR4 outbreak. It must be taken into account that the disease must be associated with the symptoms in plants. Detection in soil and water samples is useful for improving technology and decision-making for containment, but is not recommended for an official disease incursion declaration.
- During an incursion, there will be demand for planting material, especially when resistant clones are available. In this case, due caution must be exercised. Certified plant material should be available, and further discussion on this topic is needed.
- The presence of the disease brought a unique opportunity to formalize and strengthen the Regional Platforms for the diagnosis of Musaceae pests and diseases. This can lead to better synergies and coordination even beyond Musa pests.

Dr Dita highlighted the importance of regional platforms for the development of: a regional DNA bank of quarantine and regionally important pathogens; harmonized and validated protocols with continuous updating of processes; and coordinated validation of the decision-making process by national and regional plant protection organizations (NPPOs / RPPO) among other topics.

Dr Dita commended the efforts carried out by institutions collaborating with Bioversity International in Colombia, Peru and around the world and concluded his intervention.

Dr. Peng Jun presented an overview of Fusarium TR4 from China's perspective. China is the world's third largest producer of bananas. The first incursion of TR4 into the country was back in 2001, when the disease was identified in Guangdong province. Unfortunately, nowadays the disease is present in all the producing areas of the country.

Dr. Jun provided an overview of the symptoms of the disease and emphasized the need for an early and accurate detection in order to ensure sustainable production of bananas. According to the scientist, there are no effective measures to control TR4 once banana plants have been infected; he then highlighted that only a few resistant cultivars are known.

Dr. Jun presented in detail the multiple methods used by the Chinese Academy of Sciences of Tropical Agriculture (CATAS), to detect TR4 and subtropical race 4 (STR4). The most used method by the Academy is the loop-mediated isothermal amplification (LAMP) based on two internal primers: FIP and BIP. The LAMP method — followed by real-time PCR —is used by the institution for the qualitative and quantitative detection of TR4 in infected plants as well as in soil samples.

Dr. Jun compared the LAMP method with the PCR and emphasized that LAMP is faster, simpler, and more effective than PCR.

According to the researcher, CATAS has developed two (2) varieties of banana resistant to TR4. They are Baodao banana varieties: with red stems and buds and Lantianhuang banana varieties: with green stems and buds. CATAS is conducting trials aiming the development of new resistant germplasm to TR4. The center also conducts research on biological control, soil health and on the precise management of water and fertilizers for TR4 management.

For more information on the World Banana Forum and its activities in TR4, visit: http://www.fao.org/tr4gn/en/

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