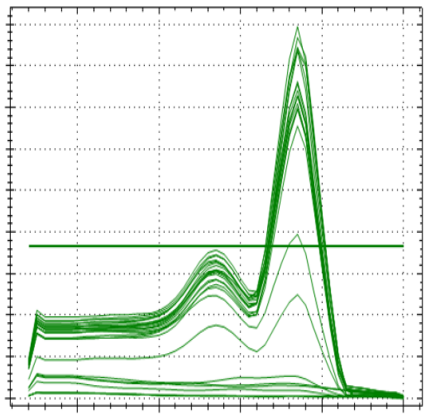
Experiences and challenges of *Fusarium oxysporum* f. sp *cubense* Tropical Race 4 diagnostic in banana crops in Colombia



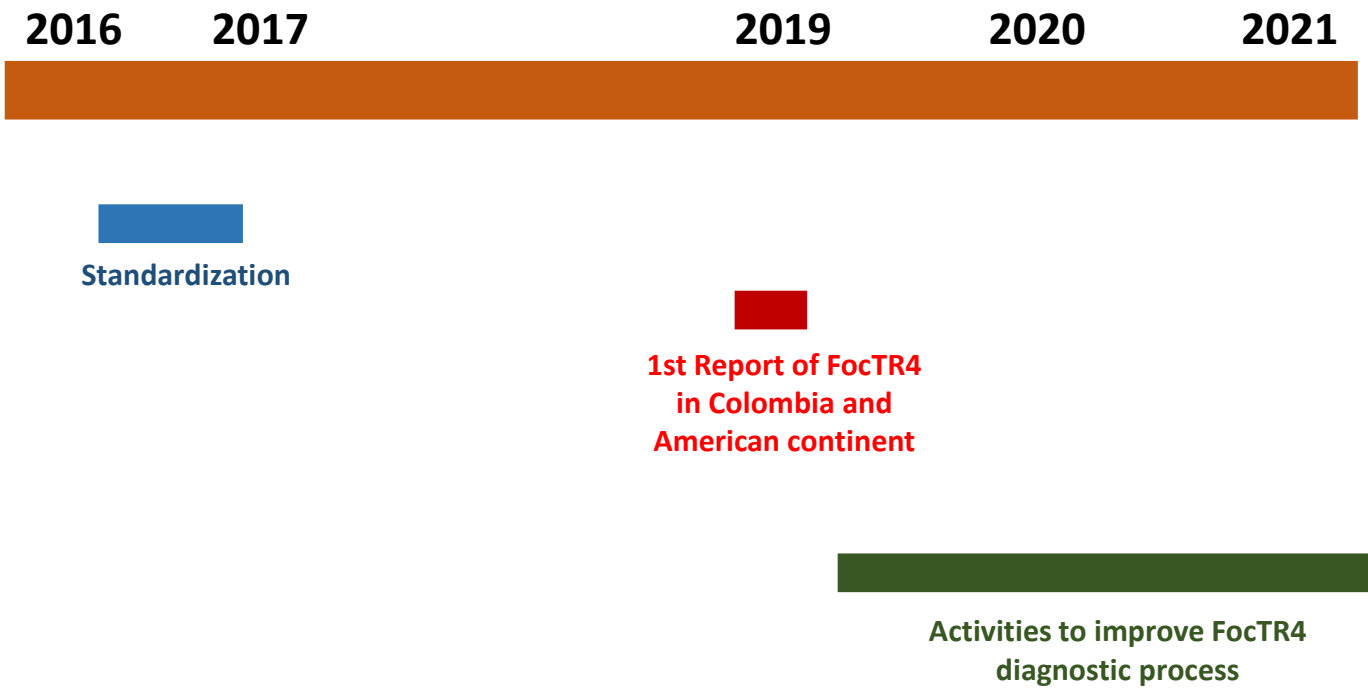
Dirección Técnica de Análisis y Diagnóstico Agrícola

Subgerencia de Análisis y Diagnóstico

Instituto Colombiano Agropecuario

By Mariluz Ayala Vásquez

Background and preliminary process of *Foc*TR4 detection



2016-2017: Memories

Early activities for *Foc*TR4 detection in Colombia

2016: Training (Banana Research Group- Wageningen University)

2016-2017: Development and standardization of FocTR4 detection method in ICA´s laboratories

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CIB Corporación para Investigaciones Biológicas

WAGENINGEN UNIVERSITY & RESEARCH

augura asociación de bananeros de colombia

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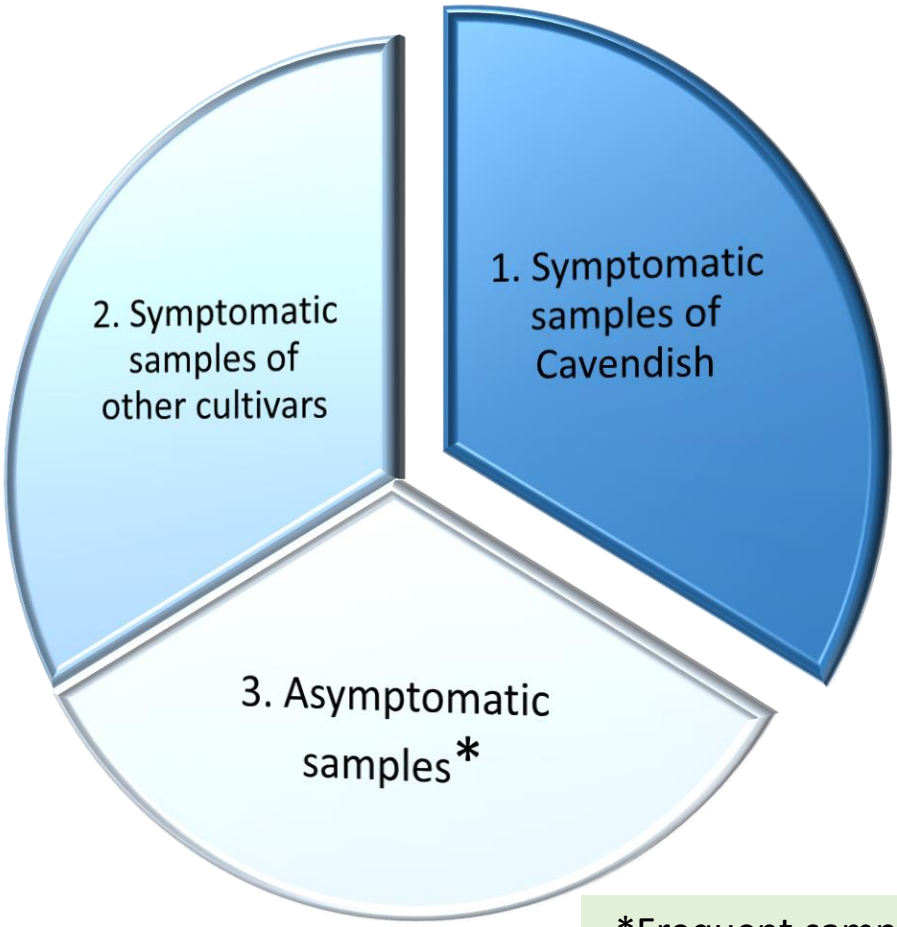
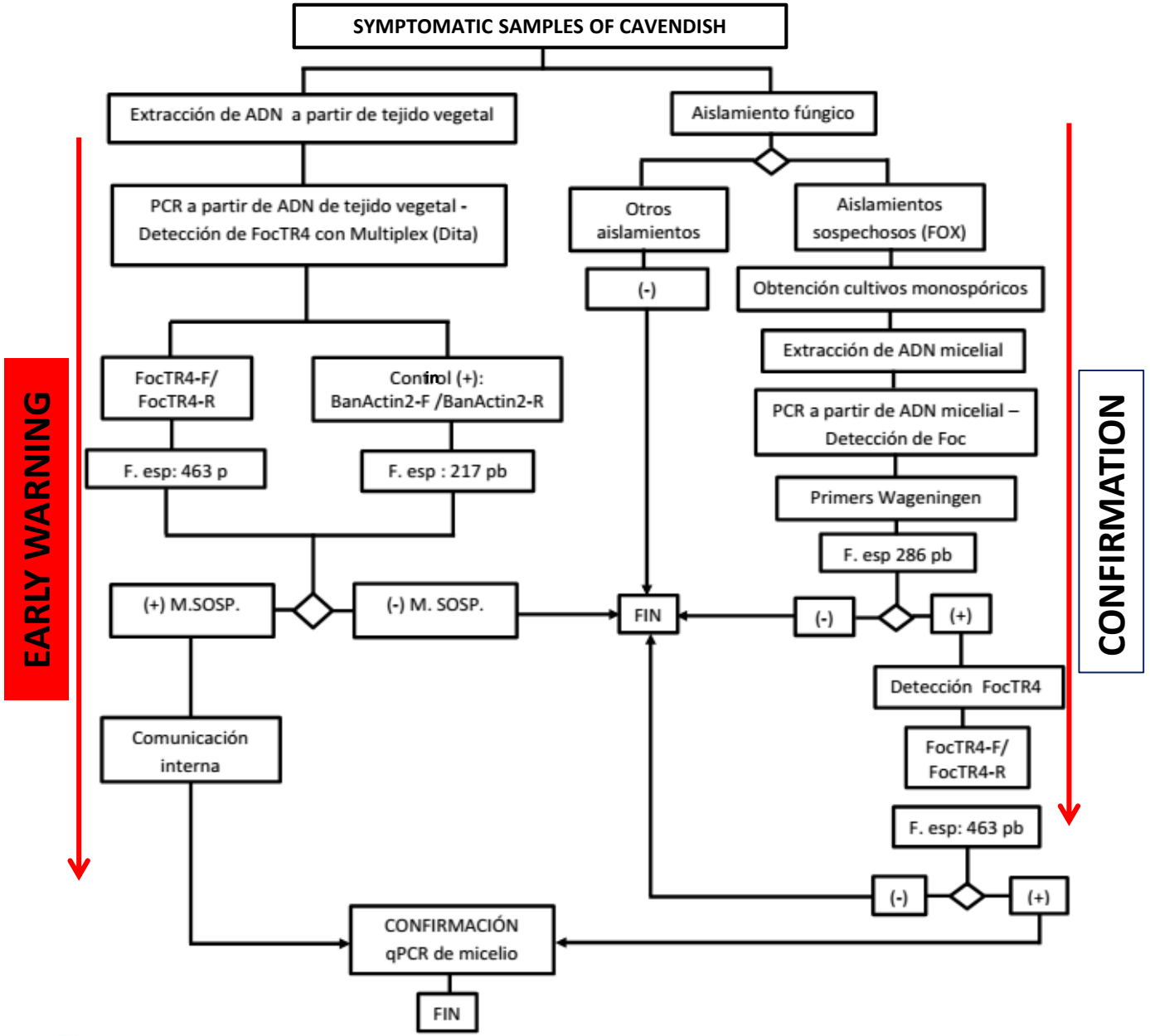
Preliminary aspects for the FocTR4 detection method

The procedure was initially determined according to the type of sample to be processed!

Generation of different alarm levels



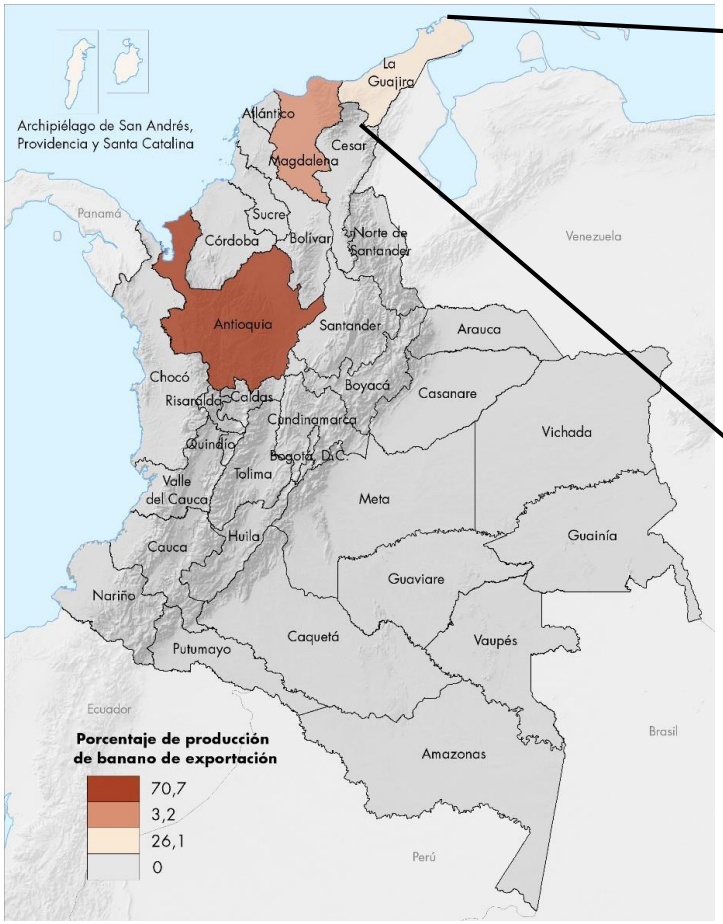
*Frequent samples

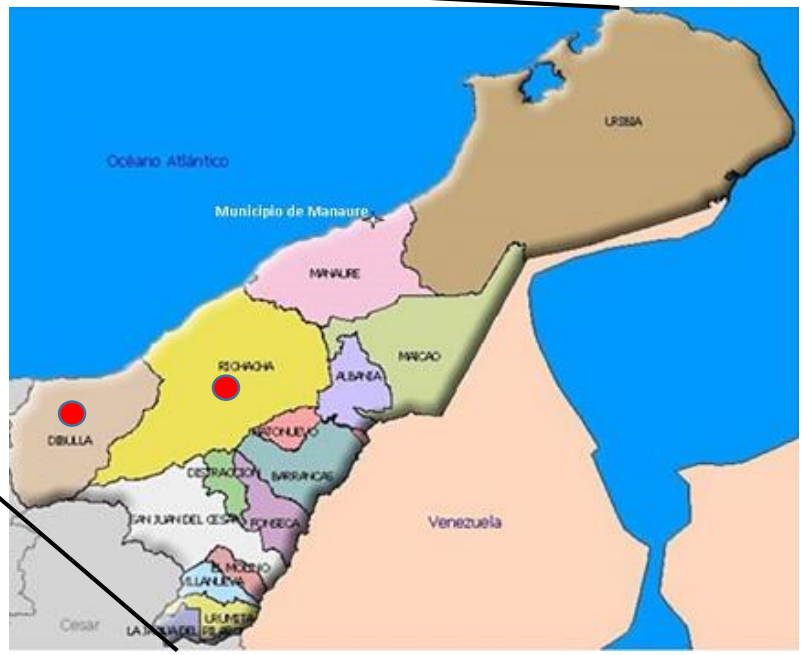

SYMPTOMATIC SAMPLES OF CAVENDISH
Extracción de ADN a partir de tejido vegetal
Aislamiento fúngico
PCR a partir de ADN de tejido vegetal - Detección de FocTR4 con Multiplex (Dita)
Otros aislamientos
Aislamientos sospechosos (FOX)
(-)
Obtención cultivos monospóricos
Extracción de ADN micelial
FocTR4-F/ FocTR4-R
Control (+): BanActin2-F /BanActin2-R
PCR a partir de ADN micelial – Detección de Foc
F. esp: 463 p
F. esp : 217 pb
Primers Wageningen
F. esp 286 pb
(+) M.SOSP.
(-) M. SOSP.
FIN
(-)
(+)
Detección FocTR4
Comunicación interna
FocTR4-F/ FocTR4-R
F. esp: 463 pb
(-)
(+)
CONFIRMACIÓN qPCR de micelio
FIN
EARLY WARNING
CONFIRMATION

2019: First report of FocTR4 in Colombia

Cavendish plants with suspicious symptoms are identified in the field

2019: First report of FocTR4 in Colombia





La Guajira County – Riohacha and Dibulla municipalities affected areas

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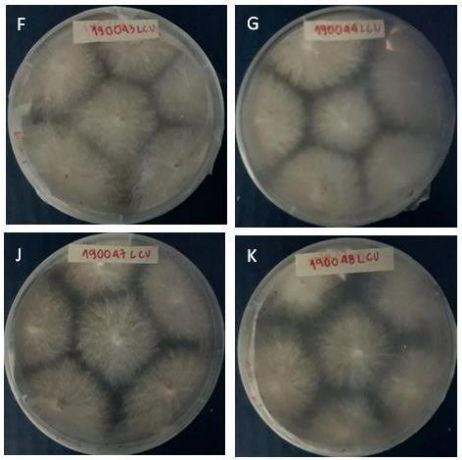
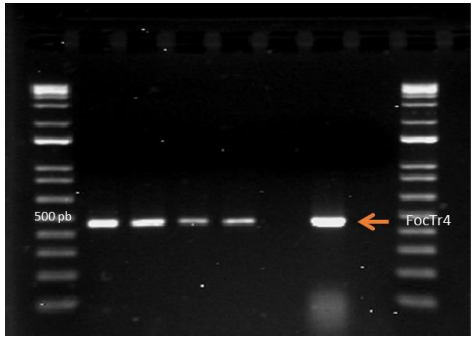
2019: First report of FocTR4 in Colombia

Official Samples

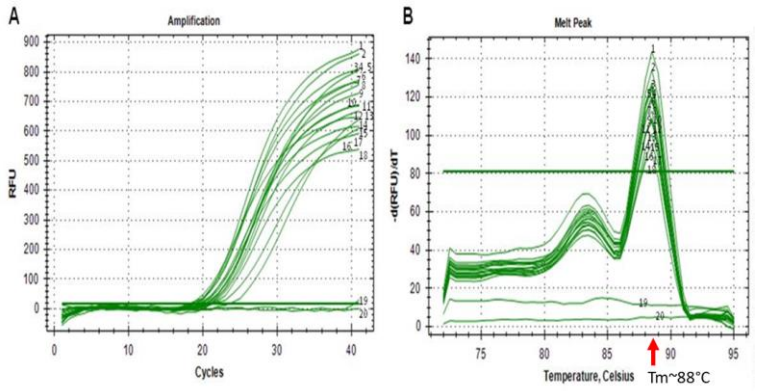
Fungus Isolation



IGS PCR end point



IGS qPCR Detection in tissue



Confirmation process of preliminary results obtained in ICA´s laboratories

International Reference Laboratory- Wageningen University
Dr. Fernando García

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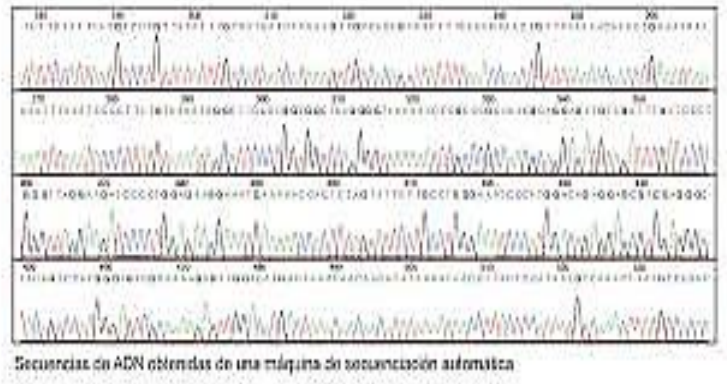
2019: First report of FocTR4 in Colombia

Implementation of complementary methodologies for confirmation process

Next Generation Sequencing - NGS



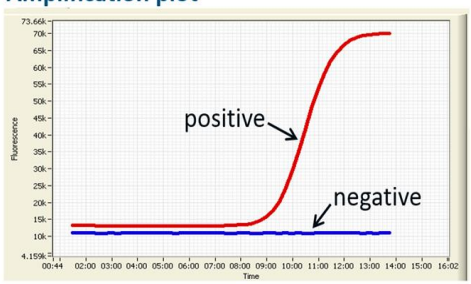
Secuencias de ADN obtenidas de una máquina de secuenciación automática

VCGs were not performed
Spend a lot of time!!

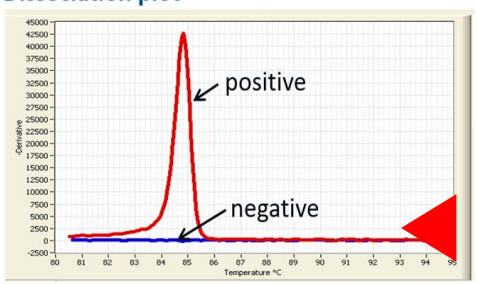
Pathogenicity tests

LAMP: Loop Mediated Isothermal Amplification

Amplification plot



Dissociation plot



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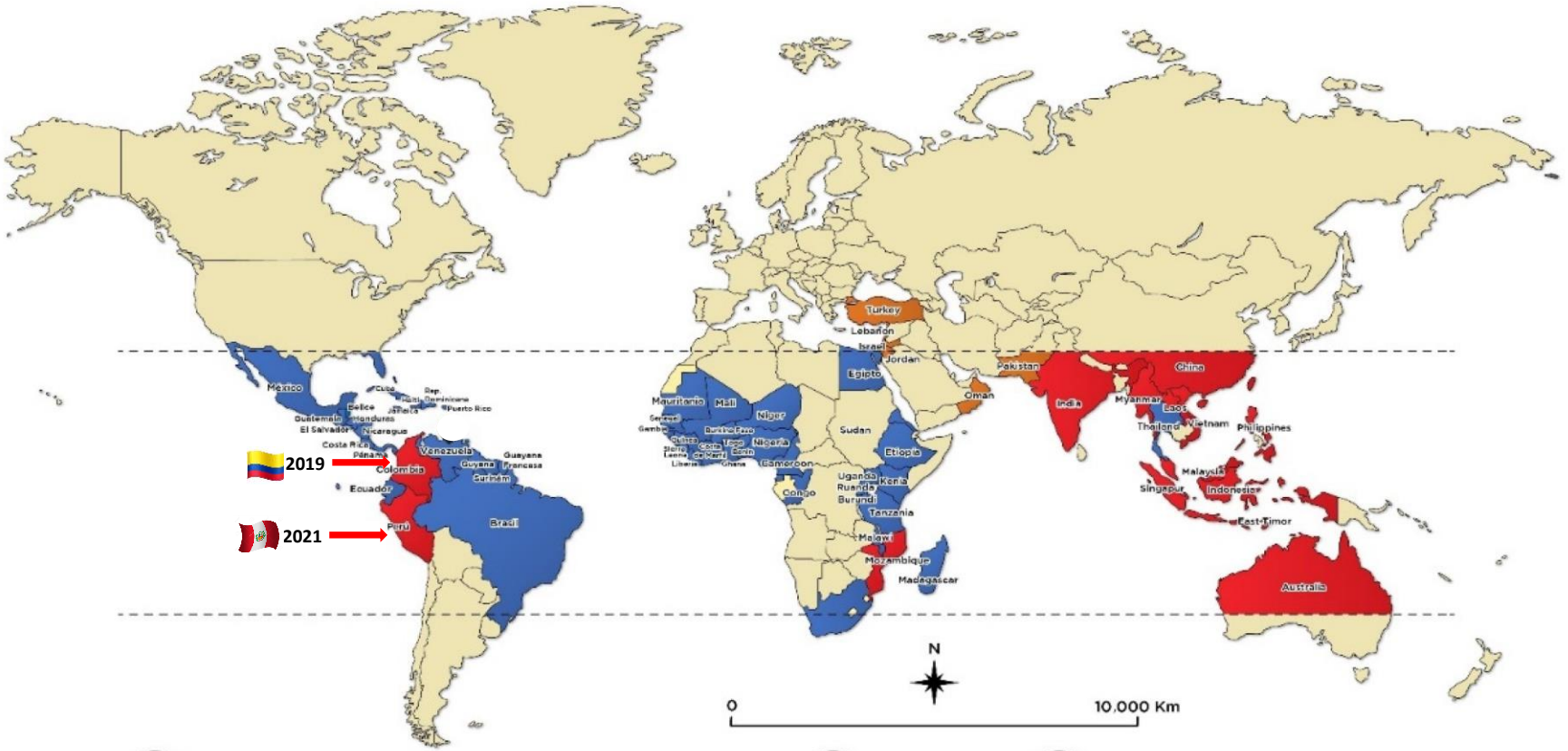
FocTR4 Current status



R1 R2

Belice	Cuba	Guayana	Mexico	Sierra leona
Benin	Ecuador	Guayana F.	Nicaragua	South Africa
Brasil	Egipto	Haiti	Niger	Surinam
Burkina F.	El Salvador	Honduras	Nigeria	Tanzania
Burundi	Etiopia	Kenia	Panama	Thailand
Cameron	Liberia	Madagascar	Puerto Rico	Togo
Congo	Gambia	Malawi	Rep. Dom.	Uganda
Costa de M.	Guatemala	Mali	Ruanda	Venezuela
Costa Rica	Guinea	Mauritania	Senegal	

TR4

Israel
Jordan
Lebanon
Mayotte
Oman
Pakistan
Turkey

TR4, R1, R2

Australia	Peru
China	Philippines
Colombia	Taiwan
India	Vietnam
Indonesia	
Laos	
Malaysia	
Mozambique	
Myanmar	

Modified from Olivares *et al*., 2021

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Activities for improving *Foc*TR4 diagnostic process

With the new FocTR4 phytosanitary status for Colombia…..

- **Diagnostic Process**

-To strengthen the FocTR4 detection methodologies: different molecular markers

-To ensure reliability and improve the results report timeframe delivery.

-To implement the guidelines describe in the ISO/IEC-17025 standard

- **Analytical capacity**

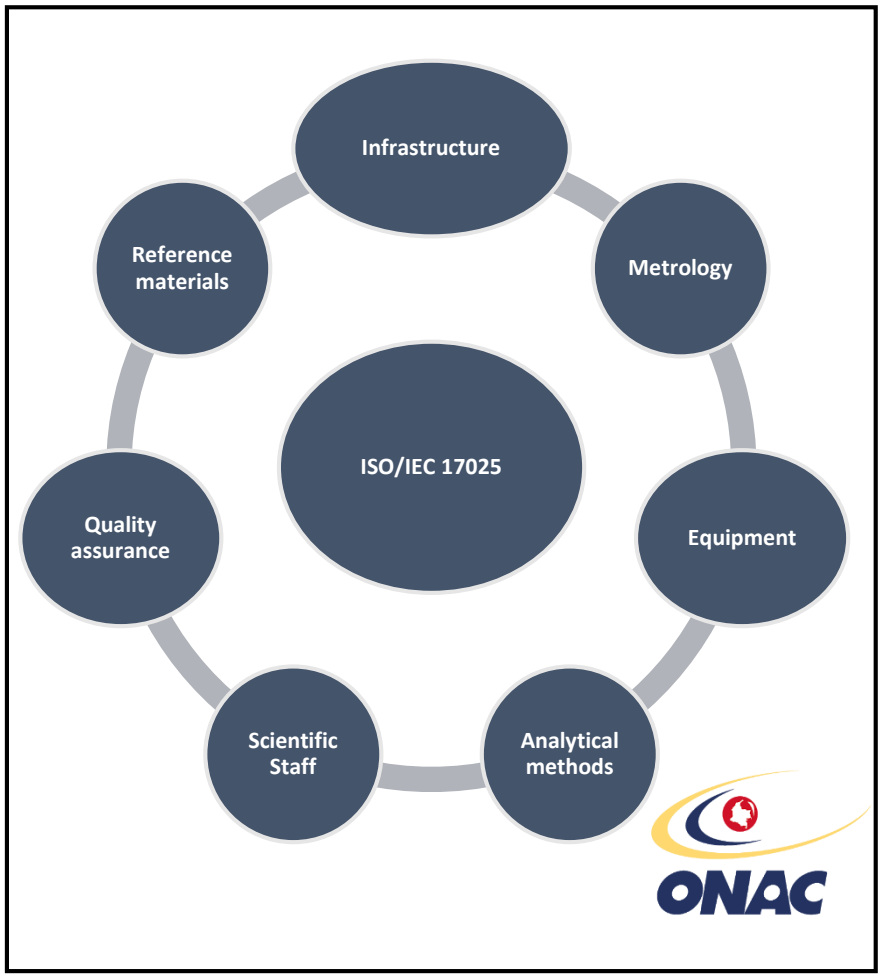
-To enable others ICA´s laboratories to face the increased demand generated by official surveillance plans.

- **Biosecurity**

-To minimize the risks of dispersion related to the mobilization of samples.

-To ensure the biological contention in the laboratories using a specific area with Good Laboratory Practice and biosecurity infrastructure.

Activities for improving *Foc*TR4 diagnostic process



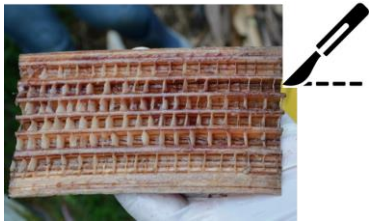
Official control beyond of the detection protocols!!.

The analytical results

For taking good decisions in the diseases management, legal actions, sanctioning processes and phytosanitary protection for the country is necessary adopt the guidelines of a quality management system.

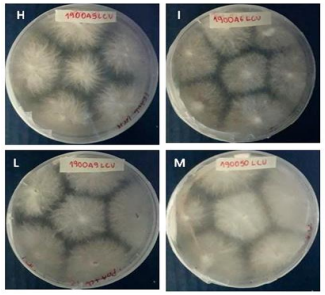
Activities for improving *Foc*TR4 diagnostic process

Samples



Recommendations
-Refrigeration, packaging reconditioning.
-Avoid excess moisture that accelerates tissue degradation processes.
-Unacceptable sample: hight state of oxidation or advanced decomposition.

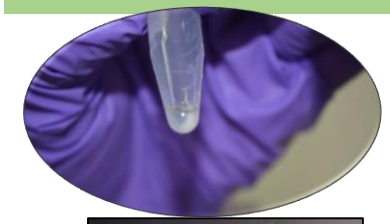
*Foc*TR4 isolation conditions



Tissue disinfection: NaClO 3%- 1 min, Sterile H2O 1 min, Drying with sterile paper

Tissue planting: 8 tissue fragments in each Petri plate, 2 repetitions, Incubation 25-28°C, Monitoring 2-3 days

DNA Extraction



Tissue/Mycelium Homogenization: by Liquid Nitrogen-zircon beads
Cell Lysis: with 320 µl Extraction Buffer (sorbitol 350 mM, Tris 100 mM pH 8.0, EDTA 5 mM, 0.2% de beta-mercaptoetanol), 320 µl Lysis Buffer (CTAB 55 mM, Tris 200 mM pH 8.0, EDTA 50 mM y NaCl 2M), 100 µl Lauroyl sarcosin 5%
Proteins and lipids separations: Washing with 1 vol chloroform: Isoamilic Alcohol
Precipitation: 1 vol Isoamilic Alcohol, 50 µl potassium acetate 5M
DNA Cleaning: Washing Ethanol (2), resuspension 100-200 µl H2O, and quantification

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Activities for improving *Foc*TR4 diagnostic process

Current scheme for the detection of *Fusarium oxysporum* f. sp. *cubense* TR4

Plant tissue analysis

Asymptomatic samples

Symptomatic samples

Fungus isolation

Symptomatic samples

DNA Extraction (CTAB Method)

qPCR Analysis / FocTR4-IGS

Endpoint PCR *Foc* detection with *CWF/CWR* (Islam, 2015)

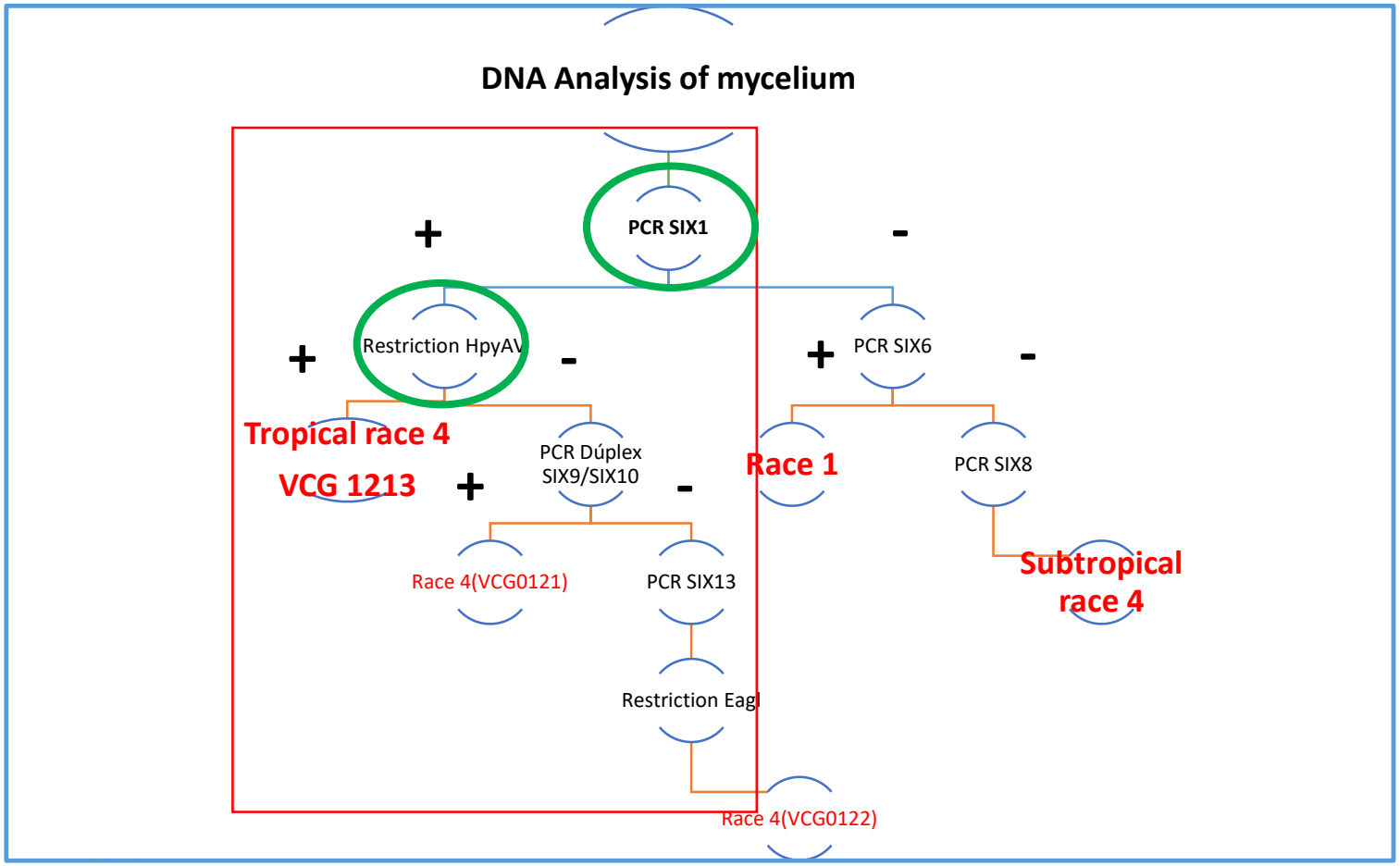
Endpoint PCR / FocTR4 detection with IGS FocTR4F/FocTR4R (Dita *el al* 2011)

SIX Gene Analysis with Endpoint PCR

Activities for improving *Foc*TR4 diagnostic process

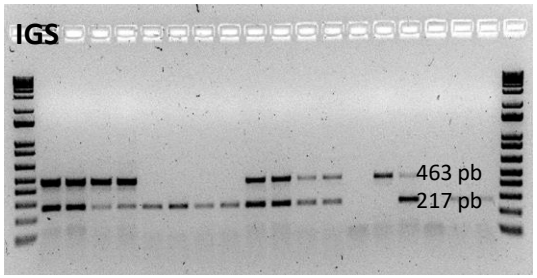
FocTR4-PCR Detection (Carvalhais *et al.,* 2019)

* Flowchart SIX gene analysis as a complementary protocol for FocTR4 detection from mycelium



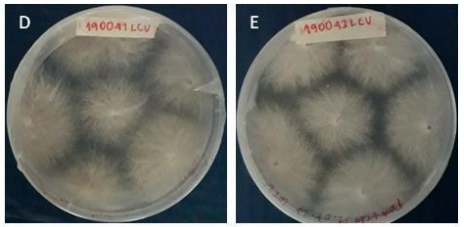
Activities for improving *Foc*TR4 diagnostic process

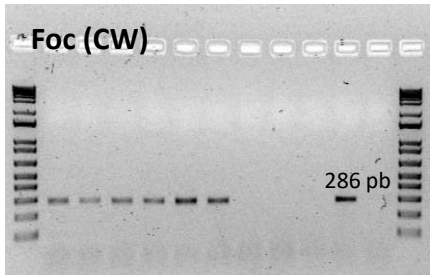
Primers	Sequence 5' - 3'	Fragment size (pb)	Gen Target	References
FocTR4-F	CACGTTTAAGGTGCCATGAGAG	463	IGS	Dita *et al*. (2010); Dita *et al*. (2011)
FocTR4-R	GCCAGGACTGCCTCGTGA			
BanActin2-F	ACAGTGTCTGGATTGGAGGC	217	Banana's Actin Gen (Internal control)	Dita *et al*. (2010)
BanActin2-R	GCACTTCATGTGGACAATGG			
CWF1	CCTGATACCCAGACGGCTAA	286	Putative protein	Islam (2015)
CWR1	CTGTCGGCTTCACCGTTATT			
SIX1_266_F	GTGACCAGAACTTGCCCACA	266	SIX Gen	Carvalhais *et al*. (2019)
SIX1_266_R	CTTTGATAAGCACCATCAA			
ITS-4	TCCTCCGCTTATTGATATGC	575	ITS	White *et al*., 1990
ITS-5	GGAAGTAAAAGTCGTAACAAGG			

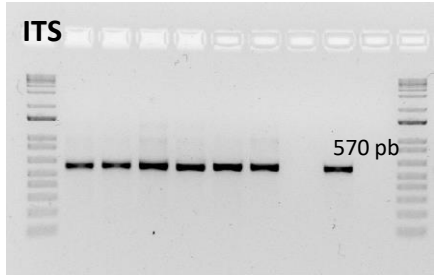


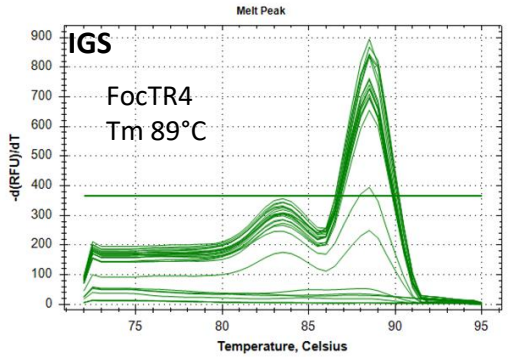
PLANT TISSUE

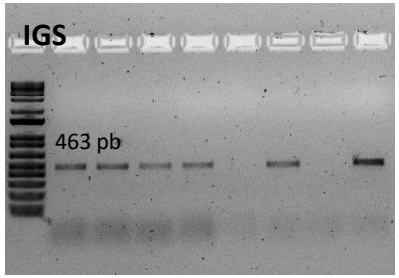
MYCELIUM

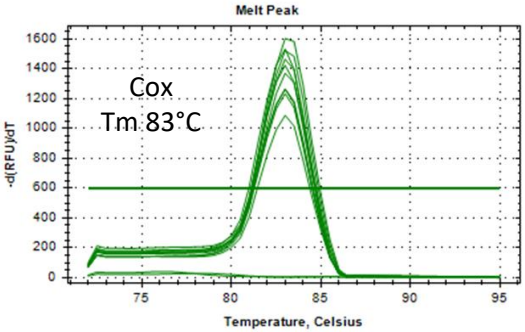


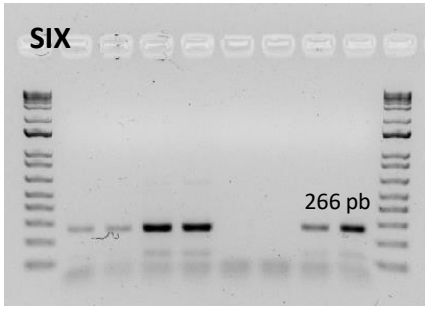


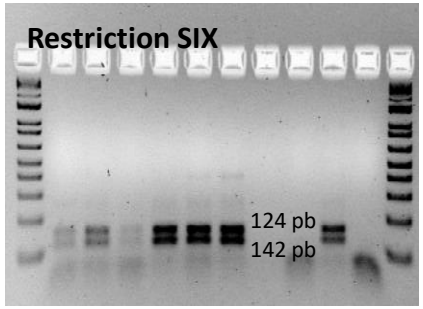












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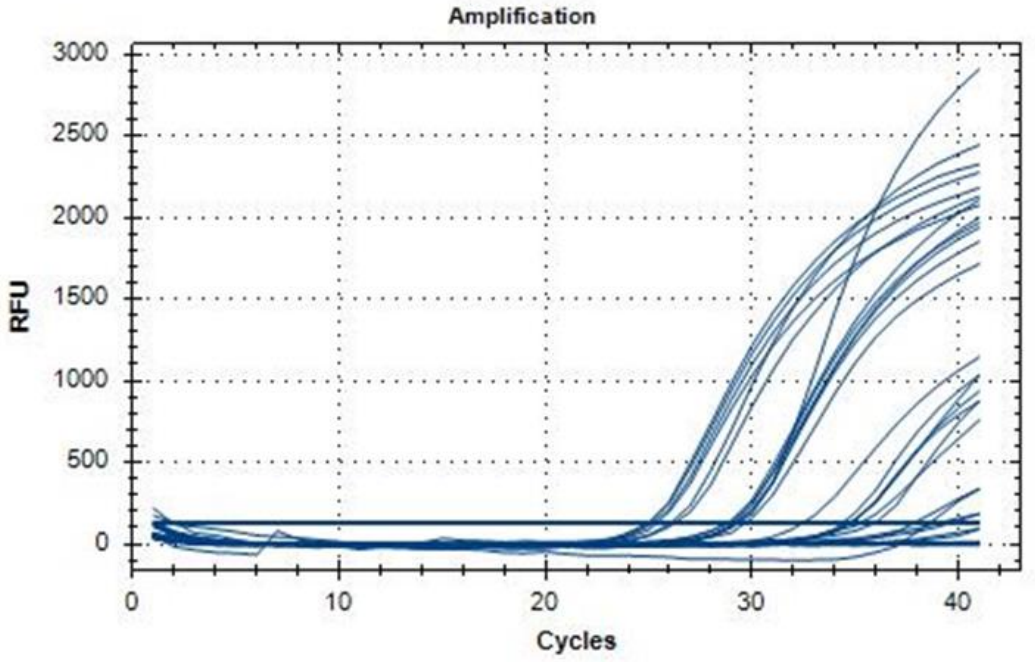
Activities for improving *Foc*TR4 diagnostic process

Additional FocTR4 detection Methodology

Primers / Probe	Sequence 5' - 3'	Gen Target	References
FWB-TR4 F	CGGTCTCGGCCAAATCTGATT	Hypothetical protein	Aguayo *et al*. (2017)
FWB-TR4 R	ACGACTTATCTAGCGGTTGATGTG		
FWB-TR4 P	ACCCTTCAACTCCACTCGATCGCA		



Activities for improving *Foc*TR4 diagnostic process

… Additional activities carried out by ICA

Andean Community- CAN project: Regional standardization of the diagnosis of *Fusarium oxysporum* f. sp *cubense* Tropical Race 4 (FocTR4)

-Workshop capacities in FocTR4 diagnostic- for CAN countries

-Standardization of diagnostic protocols

-Proficiency test: quality assurance to verify technical competence of laboratories within the framework of ISO IEC17043 standard.

New Methodology evaluation

LAMP: Loop-mediated isothermal amplification

Great POTENTIAL for FocTR4 Diagnosis/ Specificity in-situ

9 August 2021 20:31

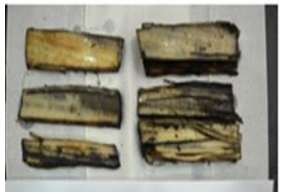
	Name		Result	Values	
1	LCV791TEJ				83.58°C
2	LCV792TEJ				83.89°C
3	LCV785TEJ				
4	HELTHPLANT				
5	SOILDIR				
6	SOILDILU				
7	BL				
8	CON+				84.47°C
9	LCV791TEJ			13:46	86.85°C
10	LCV792TEJ			17:46	86.81°C
11	LCV785TEJ			14:47	86.74°C
12	HELTHPLANT			13:52	86.86°C
13	SOILDIR				
14	SOILDILU				
15	BL				
16	CON+			14:37	87.32°C

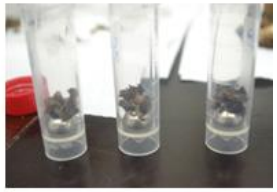
Temperature Profile

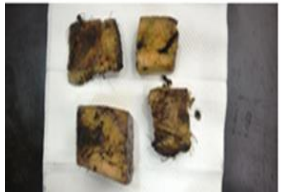
Amplification: Target 1

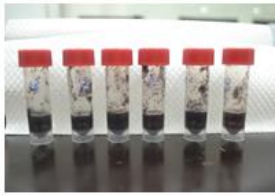
Anneal: Target 1

1 de 1









…Working

- Performance evaluation of molecular markers designed by AGROSAVIA for FocTR4 detection .

- ICA: Selection of molecular markers by comparative evaluation of attributes for further validation

- Development and implementation new methods for FocTR4 in soil.

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Augura
ASBAMA

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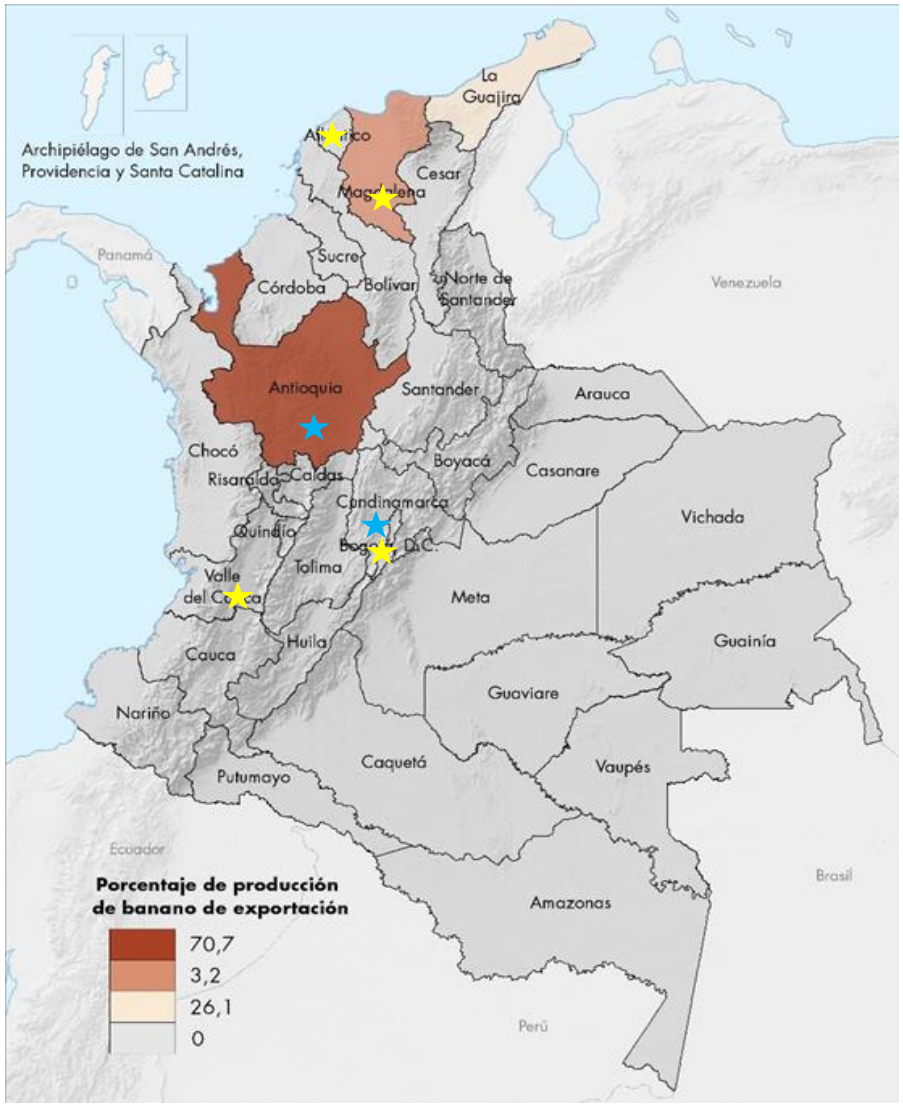
Analytical capacity

2017

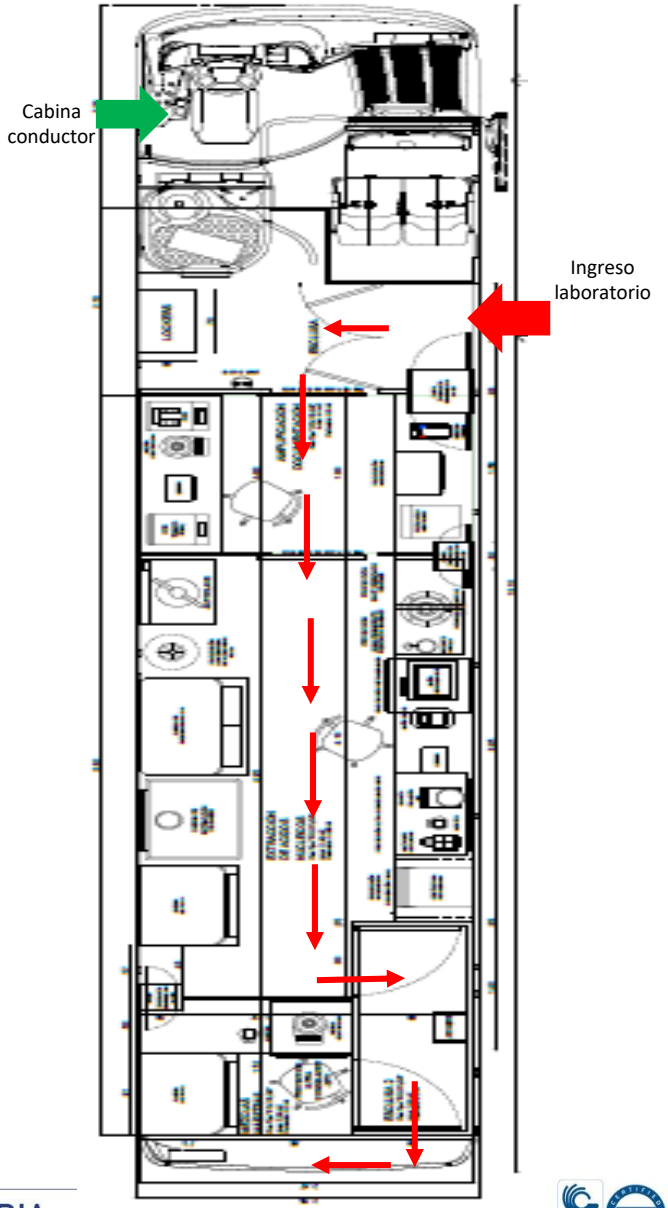
2 Authorized Laboratories

2021

6 Authorized Laboratories



Mobile Laboratory



BIOSECURITY PRACTICES IMPLEMENTED

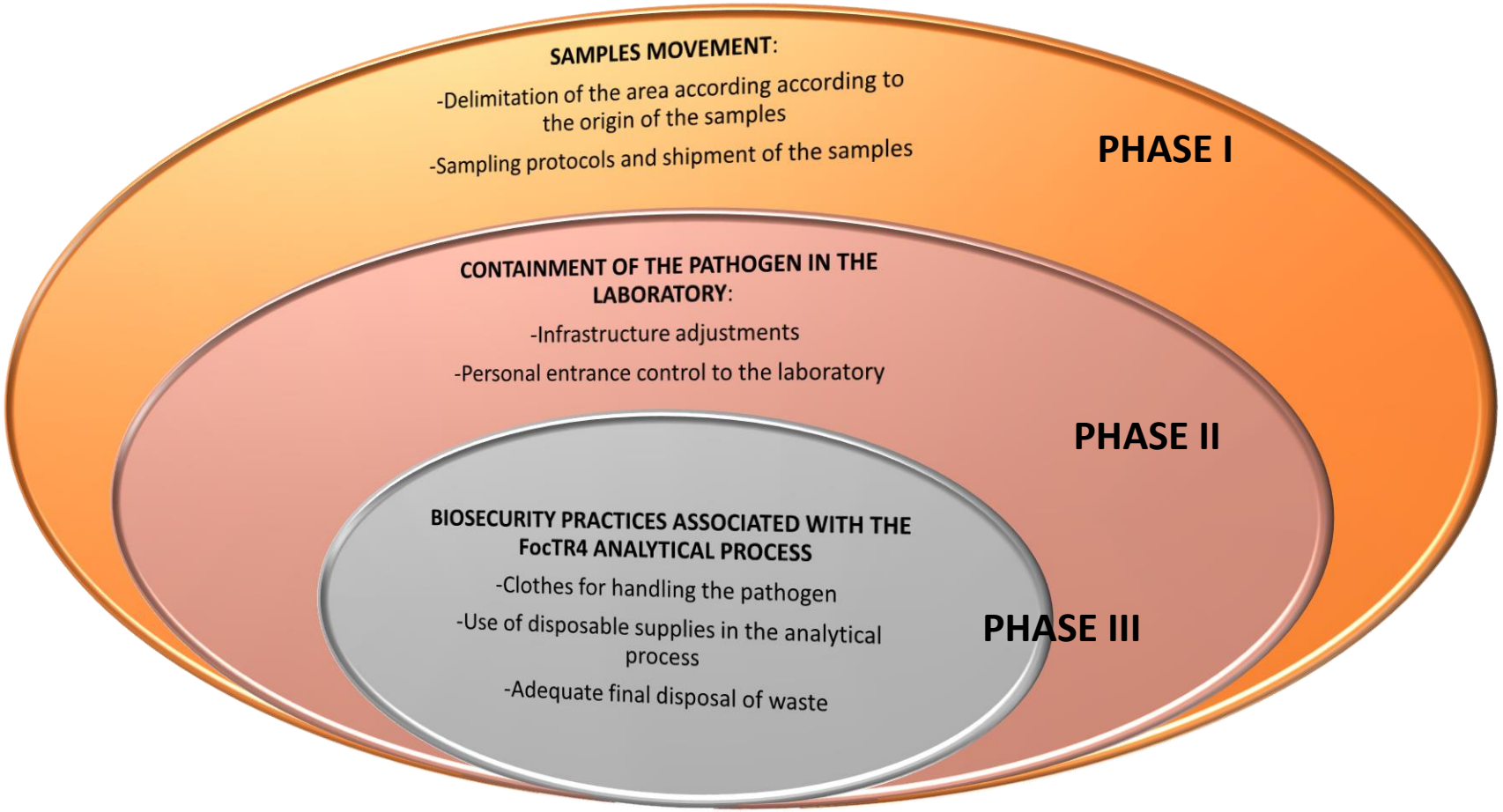
1. To establish protocols for taking, packaging and sending samples to the laboratories.

1. To manage the samples shipment according to the origin and risk of the area sampling.

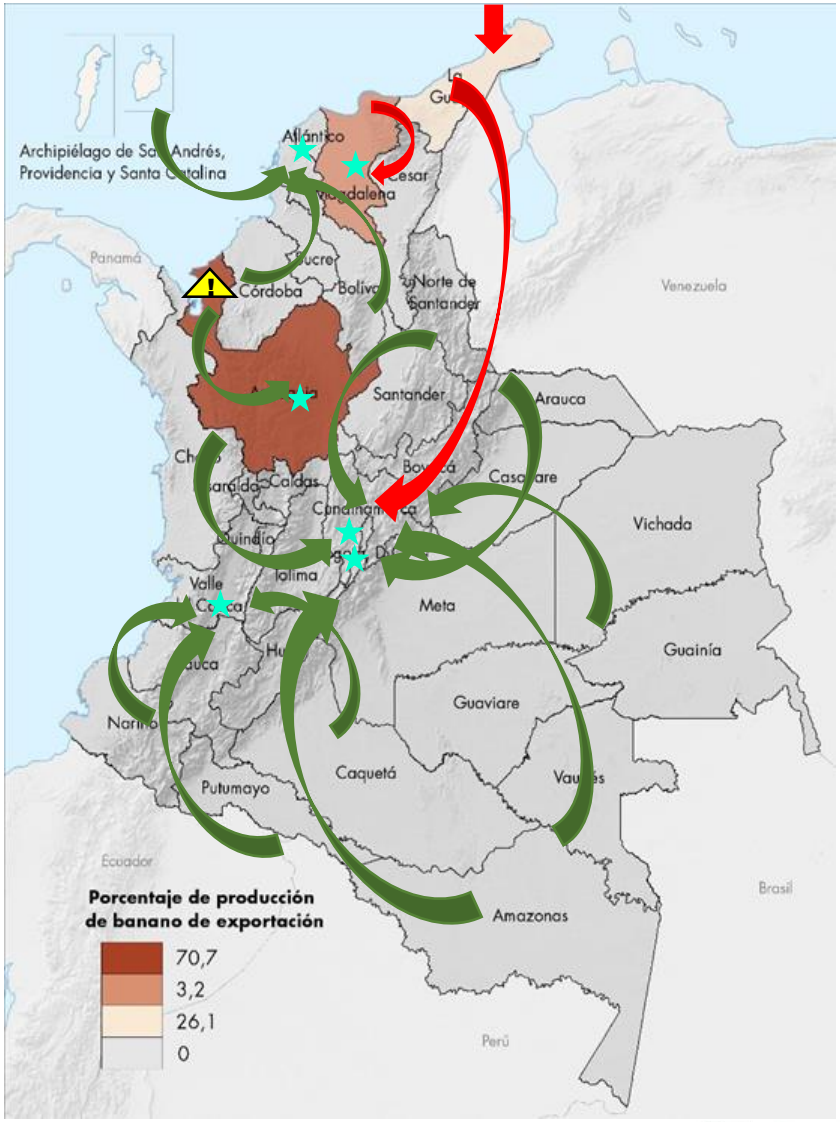
2. To define and build contention areas inside of the laboratories.

3. To design of biosecurity protocols for sample handling, and waste final disposition.

BIOSECURITY PRACTICES IMPLEMENTED



BIOSECURITY PRACTICES IMPLEMENTED



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BIOSECURITY PRACTICES IMPLEMENTED

CONTENTION AREA

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Phytosanitary requirements verification

LABORATORIO DE CUARENTENA VEGETAL-LCV
2516 masl

Diagnostic Plataform:
8 Viruses
1 Fungus
1 Bacteria

VIRUS	MÉTODOS	CONTROLES	REGIÓN GENÓMICA	REFERENCIA
Banana bunchy top virus (BBTV) Babuvirus – Nanoviridae	PCR	Fragmento Clonado	Proteína de la Master Replicasa 479 pb	Selvajaran et al (2011) Current Science. 100(1):10
Abaca bunchy top virus (ABTV) Babuvirus – Nanoviridae	PCR	Sintético	DNA S	Sharma et al (2007) Arch Virol (2008) 153: 135–147.
Cucumber mosaic virus (CMV) Cucumovirus – Bromoviridae	RT – PCR	Material Vegetal comercial para diagnóstico	Proteína de la cápside	Choi et al (1999) Journal of Virological Methods 83:67–73
Banana bract mosaic virus (BBrMV) Potyviridae - Potyvirus	RT – PCR	Sintético	Proteína de la cápside	Iskra-Caruana et al. (2008) J. Virol. Methods 153:223, 2008. (ANSES)
Banana mild mosaic virus (BanMMV) - Betaflexiviridae	RT – PCR	Sintético	Proteína de cápside	Teycheney (2005) Journal of General Virology, 86, 3179–3187.
Banana virus X (BVX) Betaflexiviridae	RT – PCR	Sintético	Proteína de la replicación	Teycheney (2005) Arch. Virol. 150: 1715-1727.
Abaca mosaic virus – (SCMV-Ab) Potyviridae – Potyvirus	RT – PCR	Sintético	Extremo 3' y Proteína de cápside	Gambley et al (2004) Australasian Plant Pathology. 33, 475–484

VIRUS	MÉTODOS	CONTROLES	REGIÓN GENÓMICA	REFERENCIA
Banana streak badnavirus (BSVs) Badnavirus – Caulimoviridae	Inmunocaptura PCR • Imové BSIMV • Mysore BSMYV • Gold Finger BSGFV • Obino l'ewai BSOLV	anses Connaître. Évaluer. protéger		Geering et al., unpublished; Le Provost et al., 2006, Journal of Virological Methods

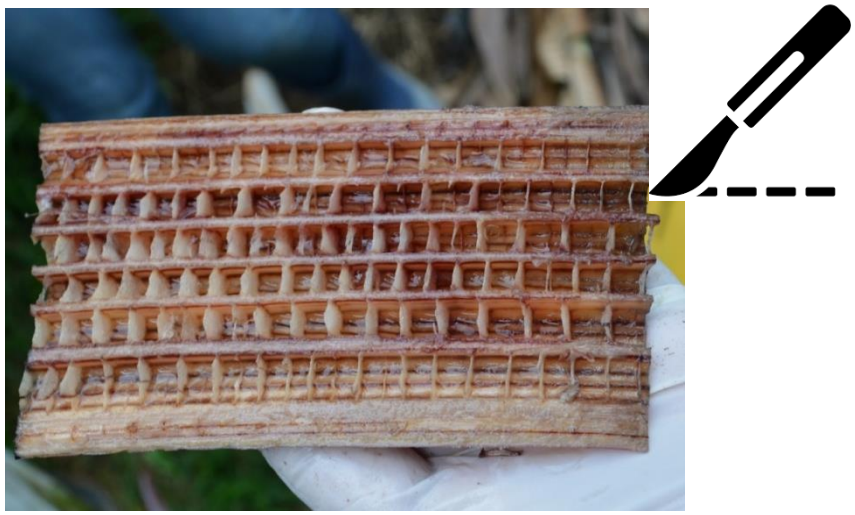
HONGOS	MÉTODOS	CONTROLES	REGIÓN GENÓMICA	REFERENCIA
Fusarium oxysporum f. sp. *cubense* Raza 4 Tropical *Foc* R4T (Asintomáticas)	Aislamiento del hongo, PCR y qPCR	Hongo purificado		

BACTERIA	MÉTODOS	CONTROLES	REGIÓN GENÓMICA	REFERENCIA
Ralstonia solanacearum Raza 2	PCR duplex	Bacteria purificada	Filotipo II patogénicas del Moko (secuevar IIB-4) y su variante fitopatológica no patogénica a musáceas(IIB-4NPB)	

THANK YOU!!

Receipt of samples

Necrosis in vascular bundles



-Refrigeration, packaging reconditioning.
-Avoid excess moisture that accelerates tissue degradation processes.

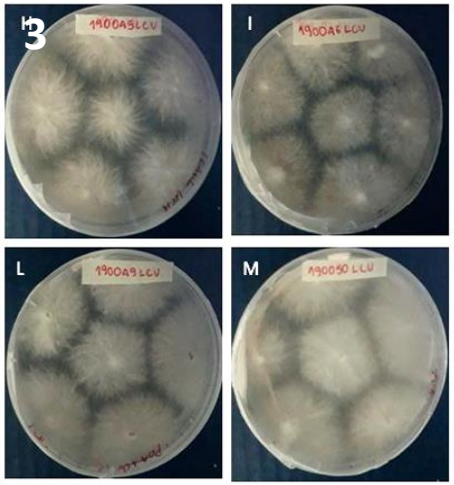
-Unsuitable sample: in a state of oxidation or advanced decomposition.

Fotos: LDFAN, ICA 2016

Isolation

Disinfection of tissue :
-NaClO 3%- 1 min
-Sterile H2O 1 min
- Drying with sterile paper

Tissue planting :
-8 explantes
-2 repetitions
-Incubation 25-28°C
-Monitoring 2-3 days



Obtaining isolates :
-DNA Extraction

DNA Extraction

1. Tissue maceration/mycelium(100-200 mg)

Liquid Nitrogen
TissueHomogenizer- zircon beads

2. Cell Lysis: Incubation 1h - 65°C

320 µl Extraction Buffer (sorbitol 350 mM, Tris 100 mM pH 8.0, EDTA 5 mM, 0.2% de beta-mercaptoetanol)

320 µl Lysis Buffer (CTAB 55 mM, Tris 200 mM pH 8.0, EDTA 50 mM y NaCl 2M)

100 µl Lauroyl sarcosin 5%

7. Quantification

3. Separation of proteins and lipids

Washing with 1 vol
chloroform :Isoamilic Alcohol

6. Resuspension

100-200 µl H_2O

5. DNA Cleaning

Washing Ethanol (2)

4. Precipitation

1 vol Isoamilic Alcohol
50 µl potassium acetate 5M