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A droplet digital PCR assay for the detection of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4

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ABSTRACT

Fusarium wilt of banana, caused by the pathogen *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc* TR4) is a threat for food security as it affects global banana industry. The development of early detection strategies of *Foc* TR4, from asymptomatic tissues with low inoculum concentration and environmental samples is an indispensable prerequisite to restrict the pathogen spread and minimize the environmental impact by eradicating an area with accurate data of the pathogen's presence. In this study, three different PCR technologies (conventional PCR, qPCR and ddPCR) were evaluated for *Foc* TR4 detection in complex environmental samples. We report the development of a sensitive and specific ddPCR primer/probe set and protocols that can be used as a tool for *Foc* TR4 detection from plant tissue without prior DNA extraction, symptomatic and asymptomatic plant tissue, drainage waters, footbaths and soil samples.

Palabras clave: *Fusarium* wilt; banana; drainage waters, footbaths, droplet digital PCR, phytosanity.

RESUMEN

La marchitez por *Fusarium* del plátano, causada por el patógeno *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc* TR4) es una amenaza para la seguridad alimentaria, ya que afecta la industria bananera mundial. El desarrollo de estrategias de detección temprana de *Foc* TR4, a partir de tejidos asintomáticos con baja concentración de inóculo y muestras ambientales, es un prerrequisito indispensable para restringir la diseminación del patógeno y minimizar el impacto ambiental erradicando un área con datos precisos de la presencia del patógeno. En este estudio, se evaluaron tres tecnologías de PCR diferentes (PCR convencional, qPCR y ddPCR) para la detección de *Foc* TR4 en muestras ambientales complejas. Informamos del desarrollo de un conjunto de cebadores/sondas y protocolos ddPCR sensibles y específicos que pueden utilizarse como herramienta para la detección de *Foc* TR4 a partir de tejido vegetal sin extracción previa de ADN, tejido vegetal sintomático y asintomático, aguas de drenaje, lavapiés y muestras de suelo.

Keywords: marchitez por *Fusarium*; plátano; agua de drenaje, lavapiés, PCR digital de gotas, Fitosanidad.

INTRODUCTION

Fusarium oxysporum f. sp. *cubense* Tropical Race 4 (*Foc* TR4) is the causal agent of banana Fusarium wilt. Once the pathogen is established in a plantation there are different factors that contribute to make difficult to control the disease, among them the ability of the fungus to grow saprophytically in plant debris, the production of chlamydospores, which allow the fungus to remain dormant in the soil for up to 30 years, the late appearance of symptoms in affected plants delaying a timely diagnosis, the high adaptability of the pathogen to diverse soil conditions and the polycyclic nature of the disease (Ploetz et al., 2015). Therefore, the development of early detection strategies of *Foc* TR4, from asymptomatic tissues with low inoculum concentration and environmental samples is an indispensable prerequisite to restrict the pathogen spread.

Due to third generation PCR technologies such as droplet digital PCR (ddPCR), it is now possible to detect and quantify target DNA directly from environmental samples such as soil, water and plant tissue, without prior isolation of the pathogen and at very low concentrations (Giber et al. 2021). The ddPCR or third-generation PCR has some advantages over qPCR, among them that no standard curve is required for quantification, resilience to PCR inhibitory substances that can be found in environmental samples, and higher sensitivity and accuracy in the quantification of target DNA (Falzone et al., 2020).

The objectives of this study were (i) to develop a *Foc* TR4 specific ddPCR assay, (ii) to use this ddPCR assay for sensitive and accurate quantification of *Foc* TR4 in complex and environmental samples, such as asymptomatic plants, soil and water. The feasibility of the ddPCR assay was verified by testing different types of samples i) DNA of purified *Foc* TR4 isolates, ii) DNA of infected plant tissue, iii) macerated plant tissue without DNA isolation, iv) DNA of artificially infested soil samples and v) DNA from drainage waters and footbaths collected from *Foc* TR4 affected fields. A comparison among PCR, qPCR and ddPCR technologies was also performed. This document represents a summary of a previously published work.

MATERIALS AND METHODS

Plant inoculation

Fusarium oxysporum f. sp. *cubense* TR4 was provided by the Instituto Colombiano Agropecuario (ICA). Two-months old *in-vitro* propagated banana plants from

commercial cultivars Valery and Williams were transplanted into pots containing 2000 g of soil. After 13 weeks, plants were drench inoculated with 100 mL of inoculum at three different concentrations of *Foc* TR4: 1 x 10^3, 1 x 10^5 and 1 x 10^6 conidia/mL. Inoculations were performed under greenhouse conditions.

DNA extraction of fungal isolates and plant material

For fungal DNA extraction, each isolate was grown in PDB for seven days under constant agitation at 30° C. Then, the mycelium was harvested, lyophilized for 48 hours at 0,3 mbar and -50 °C, and then grinded in liquid nitrogen. DNA was extracted from 100 mg of the mycelia from each isolate using the cethyltrimethylammonium bromide (CTAB) protocol (Griffith & Shaw, 1998).

For symptomatic and asymptomatic plant tissue sampling, banana fields affected with *Foc* TR4 were visited. A total of 19 symptomatic plants from 12 outbreaks in three TR4-affected fields were collected. Samples of rhizomes from Cavendish banana cultivars with external Fusarium wilt symptoms were collected. Additionally, 111 samples of asymptomatic plants located until eight meters away from 12 different symptomatic plants were collected. Finally, DNA was extracted from 300 mg of grinded tissue as reported by Daire and colleagues (Daire et al., 1997).

For plants artificially inoculated with *Foc* TR4, samples of rhizomes from Valery and Williams cultivars were collected at 70 days post-inoculation (dpi), when symptoms in rhizomes were detected for plants inoculated with 1 x 10^6 conidia/mL, and total genomic DNA was extracted as mentioned above.

Pathogen detection using PCR, qPCR and ddPCR

For pathogen detection, primer set RT_13.16_F2.5 and RT_13.16_R2.5 previously reported as specific to *Foc* TR4 was used (Matthews et al., 2020). Additionally, a FAM/BHQ-1 nova labeled probe on this *Foc* TR4-specific region for ddPCR purposes was designed using RealTimeDesign™ (https://www.biosearchtech.com/). The primers/probe used in this study were named ddPCR_13.16_F2.5, ddPCR_13.16_R2.5 and Probe_ ddPCR_13.16.

RESULTS AND DISCUSSION

A previous study has assessed the detection and quantification of *Foc* TR4 by qPCR using the PCR_13_16 marker in infected banana plants and artificially

inoculated water and soil samples (Matthews et al., 2020). Therefore, we focused our efforts on the development of a ddPCR methodology based on the same target DNA, to detect and quantify *Foc* TR4 in artificially inoculated samples, asymptomatic plant tissues, drainage waters, footbaths and soils artificially inoculated. First, we confirmed that the primer set ddPCR_13_16 is specific for F*oc* TR4 isolates. Then, we compared the LOD among PCR, qPCR and ddPCR on DNA from *Foc* TR4 isolates, and demonstrated that ddPCR offers a significant advantage, presenting a LOD with one order of magnitude lower than qPCR and PCR (Figure 1. Additionally, ddPCR was the only method capable of detecting *Foc* TR4 from macerated banana plants without a previous DNA isolation step. This result could be important, as DNA extractions are time-consuming activities to implement early pathogen detection methods based on molecular biology. Thus, the ddPCR assay developed in this study could speed up the *Foc* TR4 identification where required.

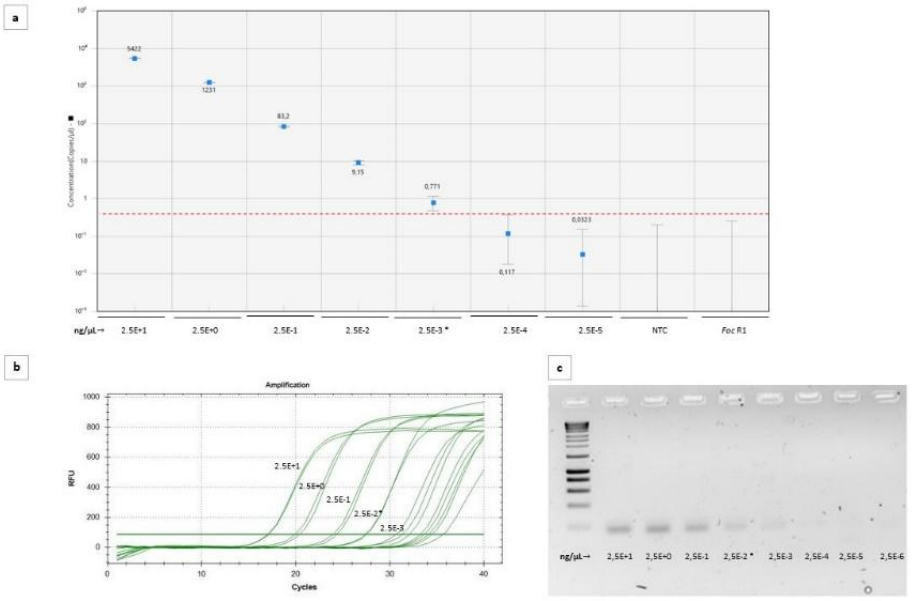

Figure 1. Comparison of level of detection (LOD) among ddPCR, qPCR and conventional PCR.

After the analysis of serial dilutions of *Foc* TR4 genomic DNA with the three technologies, ddPCR presented the lowest LOD (2,5 x 10^{-3} ng/µL) [a], followed by qPCR [b] and conventional PCR with a LOD value of 2,5 x 10^{-2} ng/µL [c].

Biosecurity measures, early detection and eradication of outbreaks are the main ways to prevent the spread of *Foc* TR4. To strengthen the containment and field monitoring activities against *Foc* TR4, reliable and sensitive tools are required for early detection of *Foc* TR4 from asymptomatic plants and environmental samples. Target

DNA could be highly diluted in complex samples (i.e. asymptomatic plant samples), requiring the use of higher amounts of plant matrix DNA per PCR reaction mix. Therefore, ddPCR offers the possibility to increase the matrix DNA quantity to up to 1 µg per reaction mixture while maintaining high resistance to PCR inhibitors (Gibert et al., 2021). In this study, up to 150 ng of plant matrix DNA per PCR mixture were required for an accurate detection of *Foc* TR4 by ddPCR in asymptomatic plants artificially inoculated with 1 x 10^5 conidia/mL of the pathogen, but not at lower concentrations (1x10^3 conidia/mL). This result suggests that ddPCR cannot detect *Foc* TR4 in very low-level infected samples or that this low inoculum concentration was not sufficient for *Foc* TR4 to develop disease symptoms. Recent studies reported that the minimum threshold of inoculum concentration in soil required to infect plants is about 1 x 10^3 spores per gram of dry soil (Wen et al. 2020). Importantly, *Foc* TR4 was detected in asymptomatic plants collected from disease-affected fields (Figure 2). As shown in other pathosystems (Pucci et al., 2022), ddPCR could be applied to the detection of *Foc* TR4 in contaminated plants with low concentration of target DNA, such as asymptomatic nursery plants or asymptomatic plants in the field.

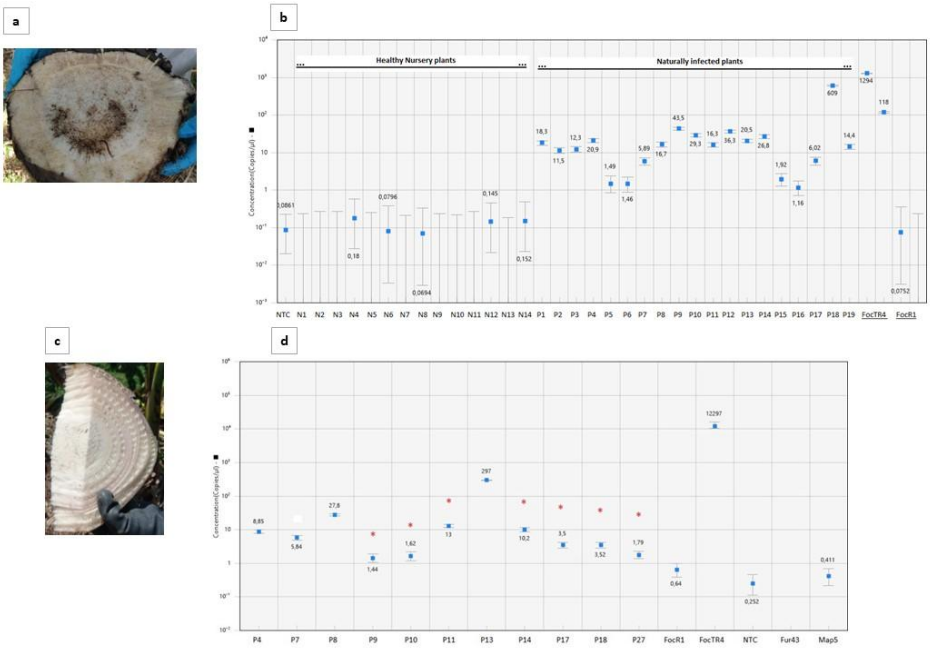

Figure 2. Detection of *Foc* TR4 in naturally infected plants**.** [a] Rhizome from symptomatic plant. [b] ddPCR from Nursery plants (N) and symptomatic naturally infected plants (P). [c] Rhizome from asymptomatic plant. [d] ddPCR from asymptomatic plants (red asterisks).

The high abundance of PCR inhibitors in environmental samples and low inoculum levels are one of the main causes of the lack of PCR-based approaches for *Foc* TR4 detection and quantification in environmental samples, such as drainage waters and soil (Matthews et al., 2020). The present ddPCR assay can quantify the number of copies of a target DNA fragment of *Foc* TR4 from drainage water samples, as low as 10^5 conidia/mL for artificially infested water samples. Additionally, we determined that DNA should be extracted as quickly as possible after collection of water samples, given that there is a substantial DNA degradation during the incubation period. For artificially inoculated soil samples, ddPCR detected *Foc* TR4 at a concentration as low as 10^3 conidia/mL per gram of soil. In contrast, soil samples should be incubated for pathogen multiplication before qPCR or ddPCR analysis.

Altogether, the results presented here demonstrate that the ddPCR can be used as a tool for detection of *Foc* TR4 directly from macerated plant tissue without prior DNA extraction. We also demonstrated that ddPCR can detect *Foc* TR4 from asymptomatic plants collected in TR4-affected fields. Additionally, artificial inoculation assays with *Foc* TR4 on drainage waters and soil samples allowed us to establish a minimal threshold for detection of *Foc* TR4 being $1x10^5$ and $1x10^3$ conidia/mL, respectively.

CONCLUSIONES

The objective of this study was to evaluate ddPCR as a new tool to detect and quantify *Foc* TR4 in complex and environmental samples for uses in research and diagnostic services. Our work demonstrates the potential of ddPCR technology to detect and quantify *Foc* TR4 from environmental and recalcitrant samples, and from samples with low target DNA concentration. To the extent of our knowledge, the current study represents the first report of *Foc* TR4 detection using ddPCR technology, and the application of ddPCR for detection of *Foc* TR4 from symptomatic and asymptomatic banana plants.

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