



Tropical Race 4 and Race 1 strains causing *Fusarium* wilt of banana infect and survive in *Heliconia* species and ornamental bananas

Einar Martínez-de la Parte · Harold J. G. Meijer ·
Mauricio Guzmán-Quesada · Claudiana Carr-Rodríguez ·
Silvia Masis-Jiménez · Luis Pérez-Vicente · Gert H. J. Kema

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Abstract *Fusarium* wilt of banana (FWB), caused by soilborne *Fusarium* lineages, is a major global threat to the cultivation of bananas. In addition to persistent chlamydospores, weeds are a reservoir of the causal agents. However, it remains unclear whether other Zingiberales species, which are grown in the same geographic regions, also can serve as hosts for strains that cause FWB. Greenhouse assays were conducted to investigate whether a Race 1 strain (pathogenic to Gros Michel banana) or Tropical Race 4 (TR4) (pathogenic to a plethora of banana varieties, including Cavendish bananas) can infect three *Heliconia* species, two ornamental banana species or *Musa textilis* (abacá). *Heliconia latispatha*, *Musa*

balbisiana, and *Musa coccinea* displayed external symptoms after inoculation with TR4, while inoculation with Race 1 caused symptoms in *H. latispatha*, *H. psittacorum*, *M. coccinea*, and *M. velutina*. Isolates were recovered from symptomatic and asymptomatic plants and were subsequently characterized and used to inoculate Gros Michel and Cavendish banana plants. They caused the typical FWB symptoms in these varieties, and the scores for discolored rhizome area were similar to those caused by the Race 1 and TR4 reference strains. These data call for a revision of the race nomenclature of FWB pathogens and adjustment of the current containment protocols.

Keywords *Heliconia* · *Fusarium odoratissimum* · TR4 · Abacá · Secondary hosts

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E. Martínez-de la Parte · G. H. J. Kema (✉)
Laboratory of Phytopathology, Wageningen University & Research, Wageningen, The Netherlands
e-mail: gert.kema@wur.nl

E. Martínez-de la Parte · L. Pérez-Vicente
Instituto de Investigaciones de Sanidad Vegetal (INISAV),
La Habana, Cuba

H. J. G. Meijer
BU Biointeractions & Plant Health, Wageningen
University & Research, Wageningen, The Netherlands

M. Guzmán-Quesada
Cropland Bioscience, San José, Costa Rica

M. Guzmán-Quesada · C. Carr-Rodríguez
Departamento de Fitoprotección, Corporación Bananera
Nacional (CORBANA S.A.), Limón, Costa Rica

C. Carr-Rodríguez
American Vanguard Company (AMVAC), Alajuela,
Costa Rica

S. Masis-Jiménez
Laboratorio de Cultivo de Tejidos, Coordinación de
Fisiología, Clima y Producción, Corporación Bananera
Nacional (CORBANA S.A.), Limón, Costa Rica

Introduction

The genus *Musa* belongs to the Musaceae family of the order Zingiberales and comprises many wild and seeded banana species as well as all seedless and therefore edible varieties. Cultivated bananas are a major staple food for millions of people in many countries and dominate the global fruit commerce (FAO, 2022; Ploetz, 2015). The genus *Musa* is divided into two sections, *Callimusa* and *Musa*, with the latter including most edible banana cultivars (Häkkinen, 2013). These are diploids or triploids exclusively originating from *Musa acuminata* (AA, $2n=22$) or from hybridizations with *Musa balbisiana* (BB, $2n=22$) (Ploetz, 2006). Only a minor group of cultivars, which included Fe'i bananas, is derived from species of the *Callimusa* section (Häkkinen, 2013). This section also includes *M. textilis* (also known as abacá) which is appreciated as a source of cordage fiber. Ornamental banana species can be found in both of the aforementioned sections and typically have relatively few fruits and are best known for their brightly colored bracts (Häkkinen, 2013; Häkkinen & Väre, 2008). Another group of ornamental species, *Heliconia* spp. also belong to the Zingiberales, but to the family of the Heliconiaceae. They are very popular neotropical ornamentals characterized by colorful inverted flowers (Gómez-Merino et al., 2018).

Fusarium wilt of banana (FWB) is a devastating vascular disease that severely impacts international banana production (Staver et al., 2020) and jeopardizes food security and livelihoods in regions that rely on banana cultivation (Fones et al., 2020; Steinberg & Gurr, 2020). The disease is caused by polyphyletic, soil-borne fungi belonging to the *Fusarium oxysporum* species complex (FOSC), which have been traditionally classified as *Fusarium oxysporum* f. sp. *cubense* (Foc). Phylogenetic analyses, have consistently shown that Foc isolates are divided into three major clades, which are further subdivided into eight to 10 lineages (Maryani et al., 2019; Mostert et al., 2022; Ordóñez et al., 2015). These lineages encompassing the various physiological races were classified as individual phylogenetic species by Maryani et al. (2019), with Tropical Race 4 (TR4) strains classified as *F. odoratissimum* and Race 1 strains comprising a suite of seven *Fusarium* spp., including *F. phialophorum*. A recent pangenome analysis, based on more than 3,800 single-copy orthologous genes (van

Westerhoven et al., 2024) and whole-genome Single Nucleotide Polymorphisms (Martínez-de la Parte et al., 2024), supports the initial species naming by Maryani et al. (2019). However, more research is required to test whether the observed diversity should lead to a new nomenclature of Foc that comprises designated lineages within the FOSC. In this study, we will use the race designation Race 1 and TR4 as representatives of the proposed species *F. phialophorum* and *F. odoratissimum*, respectively.

The FWB pathogens invade the vascular system of banana plants after root infection, obstructing water and nutrient transport ultimately leading to wilting, chlorosis, and plant death (Pegg et al., 2019). To date, three different races have been described based on the pathogenicity to reference banana cultivars, named Race 1, Race 2 and Race 4, with the latter subdivided into TR4 and Subtropical Race 4 (Pérez-Vicente, 2004; Ploetz, 2015). A pathogen population causing wilt in *Heliconia* spp., was described as Race 3 (Waite, 1963), but it is no longer considered part of *Fusarium* complex infecting banana (Ploetz, 2015). The FWB pathogens also reside and survive in non-banana plant species (Hennessy et al., 2005; Pittaway et al., 1999; Waite & Dunlap, 1953) which contributes to their long-term survival in the field. Some *M. velutina* and *M. textilis* accessions were included in germplasm screenings for resistance to TR4 (García-Bastidas, 2019; Li et al., 2015; Zuo et al., 2018), but others, such as *M. coccinea*, have never been tested. Additionally, *Fusarium* isolates infecting *M. textilis* have not been used in disease evaluations of bananas (Borines et al., 2007; Dyah Purwati & Hidayah, 2008). Since these Zingiberales spp. are grown in the same geographical areas as bananas it is crucial to understand their role in the FWB epidemiology to support improved disease management strategies. Therefore, we conducted greenhouse assays aiming to understand the potential role of *Heliconia* spp. and the aforementioned non-edible *Musa* spp., in the epidemiology of FWB pathogens.

Materials and methods

Plant material

Tissue culture plants of *Heliconia latispatha* Benth., *Heliconia psittacorum* L., *Heliconia rostrata* Ruiz &

Pav., *Musa coccinea* Andrews, *M. velutina* H. Wendl. & Drude, *M. textilis* Nee, and cv. Gros Michel (AAA) were propagated at the tissue culture laboratory of the Corporación Bananera Nacional (CORBANA S.A.), Costa Rica. Tissue culture banana plants cv. Grand Naine (AAA) were obtained from Vitropic (Saint Mathieu-de-Trévières, France). Upon arrival at Wageningen University & Research (WUR, The Netherlands), the plants were transferred from transport plastic boxes to 1L pots containing a standard soil (Swedish sphagnum peat 20%, Baltic peat 30%, garden peat 30%, beam structure 20%, grinding clay granules 40.6 kg/m³, Lime+MgO 2.5 kg/m³, PG-Mix-15–10–20 0.8 kg/m³) from the WUR-Unifarm greenhouse facility. The potted plants were then acclimatized under plastic to maintain high humidity conditions for two weeks in an environmentally controlled greenhouse compartment and thereafter grown for ~2.5 months prior to inoculation (28 ± 2 °C, 16 h light, and ~85% relative humidity). During the experiment, plants were watered daily and fertilized three times per week (NH₄⁺-1.2 mM/L, K⁺-7.2 mM/L, Ca²⁺-4 mM/L, Mg²⁺-1.82 mM/L, NO₃⁻-12.4 mM/L, SO₄²⁻-3.32 mM/L, H₂PO₄⁻-1.1 mM/L, Mn²⁺-10 µMol/L, Zn²⁺-5 µMol/L, B-30 µMol/L, Cu²⁺-0.75 µMol/L, Mo-0.5 µMol/L, Fe/DTPA-50/3%, Fe-EDDHA-50/3%, pH=5.8).

Inoculum production and inoculation methods

We used the Race 1 (*F. phialophorum*, strain CR1.1A) and TR4 (*F. odoratissimum*, strain II-5) reference strains (van Westerhoven et al., 2024) for inoculations. Strain II-5 originates from Indonesia, which is the center of diversification of bananas (Janssens et al., 2016) and a biodiversity hotspot for its pathogens (Drenth & Kema, 2021) and CR1.1A is from Costa Rica, one of the main producing and exporting countries of tropical Zingiberales ornamentals (Malakar et al., 2023). To produce inoculum, conidial suspensions were produced in flasks containing 100 mL of mung beans broth (2 g mung beans per 500 ml water) according to García-Bastidas et al. (2019). The flasks were incubated at 25°C, 150 rpm for five days, after which the concentration was adjusted to 1 × 10⁶ conidia ml⁻¹ prior to inoculation.

Inoculations were performed by wounding the roots in the soil using a soil scoop at two opposite

sides of the plant and subsequent drenching with a conidia suspension of 200 ml of 10⁶ conidia/L per pot (García-Bastidas et al., 2019). For negative controls, we drenched each pot with 200 ml of water after damaging the roots, and inoculated Grand Naine and Gros Michel plants were used as positive controls for TR4 and Race 1, respectively. During the experiment, six plants of each species were inoculated with Race 1 and nine with TR4, while three to five plants were used as negative control (Table S1). The experiment was repeated twice using a Randomized Complete Block Design.

Disease progress and diagnosis of reisolated strains from plants inoculated with Race 1 or TR4

Plants were inspected every week for any noticeable FWB symptoms compared to the non-inoculated and positive controls. Five plants per species were randomly selected 12 weeks post-inoculation, and three pieces (~1 cm²) of plant material were collected from the roots, rhizome, or corm and pseudostem of each plant. Sampled plant parts were extensively washed with demineralized water and then surface sterilized with 70% ethanol for five minutes in a laminar flow cabinet, thrice rinsed with sterile demineralized water and placed on Komada's semi-specific media for *Fusarium* spp. (Komada, 1975). After six days, small fragments of the edges of fungal colonies with *Fusarium* morphology were collected and transferred to fresh potato dextrose agar (PDA) plates.

For diagnosis, fragments of the mycelium on PDA plates (five to seven days old) were collected with a sterile toothpick and transferred into a tube containing 20 µL of dilution buffer included in the Phire Plant Direct PCR Master Mix (Thermo Scientific™, Landsmeer, the Netherlands), which does not require separate DNA extraction. The tube was vortexed for 10 s and incubated at 95°C for 5 min. After a spin-down step of 10 s, one µL of the solution without mycelia was used as template in 20-µL PCR, which contains 10 µL of the Master Mix (2X) and 0.5 µL of each primer (10mM). The PCR program comprised an initial denaturation at 98°C for 5 min, followed by 35 or 40 cycles of denaturation at 98°C for 5 s, annealing at 62°C or 55°C (Table 1) for 5 s and extension at 72°C for 20 s, and a final extension at 72°C for 1 min. We used the primers of Dita et al. (2010), and Carvalhais et al. (2019)

(Table 1) for final molecular diagnosis. The resulting amplicons were visualized after electrophoresis with a 100-bp ladder as a reference on 1.5% agarose gel by staining with ethidium bromide, and gels were photographed using the ChemidocTM MP image system (Bio-Rad Laboratories, Veenendaal, The Netherlands).

Pathogenicity of reisolated *Fusarium* isolates

From each plant species, we selected a Race 1 or TR4 isolate that tested positive with the abovementioned molecular diagnostics, and was reisolated from the uppermost colonized part of the plant, for inoculations in banana following the aforementioned protocols. Twelve-week-old plants of Gros Michel or Grand Naine were inoculated with Race 1 and TR4 isolates, respectively, using the reference strains CR1.1A (Race 1) or II-5 (TR4) as positive controls, and water-treated plants as negative controls (mock). For each re-isolate we inoculated seven Gros Michel or Grand Naine plants, in two separate experiments following a Randomized Complete Block Design. The rhizomes of the inoculated plants were cut transversally 11 weeks after inoculation, photographed and the Rhizome Discoloured Area (RDA) was calculated using ImageJ 1.52r software (National Institutes of Health, Bethesda, MD, USA). Individual RDA values were plotted using the web-tool Box-PlotR (Spitzer et al., 2014). The Kruskal–Wallis test was used to compare RDA values of Grand Naine or Gros Michel plants caused by the various isolates and Dunn's multiple comparisons test was applied for multiple comparisons of variables at a P value of <0.05.

Results

To investigate the host range of FWB pathogens among *Heliconia* spp. and the level of susceptibility of non-edible bananas used as ornamental or fiber crop, we conducted greenhouse tests where *H. latispatha*, *H. psittacorum*, *H. rostrata*, *M. coccinea*, *M. velutina* and *M. textilis* were challenged with inoculum of two reference strains for Race 1 and TR4. Throughout all the individual phenotyping experiments conducted in this study, negative controls never showed any external or internal FWB symptoms. Grand Naine plants, used as positive controls, displayed the typical FWB symptoms after inoculation with TR4 but Race 1 inoculations did not result in any external or internal FWB symptoms (Fig. 1).

All plants of non-edible *Musa* spp. showed FWB symptoms typical for susceptible responses at 12 weeks after inoculation with Race 1 (Fig. 1) with an average RDA value higher than 38% with *M. coccinea* being the most susceptible (RDA = 62%). Both *M. coccinea* and *M. textilis* were also susceptible to TR4 with average RDA values of 77.8% and 53.2%, respectively. However, 35% of the *M. velutina* plants did not show any external or internal symptoms while the remainder of the plants showed the lowest scores among the tested *Musa* spp. (Fig. 1) with an average RDA value of 3.8% at 12 weeks after inoculation.

Inoculation of the three *Heliconia* spp. with Race 1 caused wilting symptoms on *H. latispatha* and *H. psittacorum* but not on *H. rostrata*. The symptoms included foliar chlorosis or drying, weakened pseudostems that easily dislodged from the base upon pulling, similar to the symptoms of wilted *Heliconia* plants under natural conditions in the field (Castro et al., 2008; Waite, 1963). However, all Race 1

Table 1 Primers for molecular diagnosis of re-isolated *Fusarium* strains

Target	Primer name	Sequence (5'–3')	Annealing Temperature (°C)	Reference
TR4	FocTR4-F	CACGTTTAAGGTGCCATGAGAG	60	Dita et al., 2010
	FocTR4-R	GCCAGGACTGCCTCGTGA		
Elongation Factor-1 α	EF-1	ATGGGTAAGGAGGACAAGAC	60	O'Donnell et al., 1998
	EF-2	GGAGGTACCAGTGATCATGTT		
Race 1	Six6b_210-F	ACGCTTCCCAATACCGTCTGT	55	Carvalhais et al., 2019
	Six6b_210-R	AAGTTGGTGAGTATCAATGC		

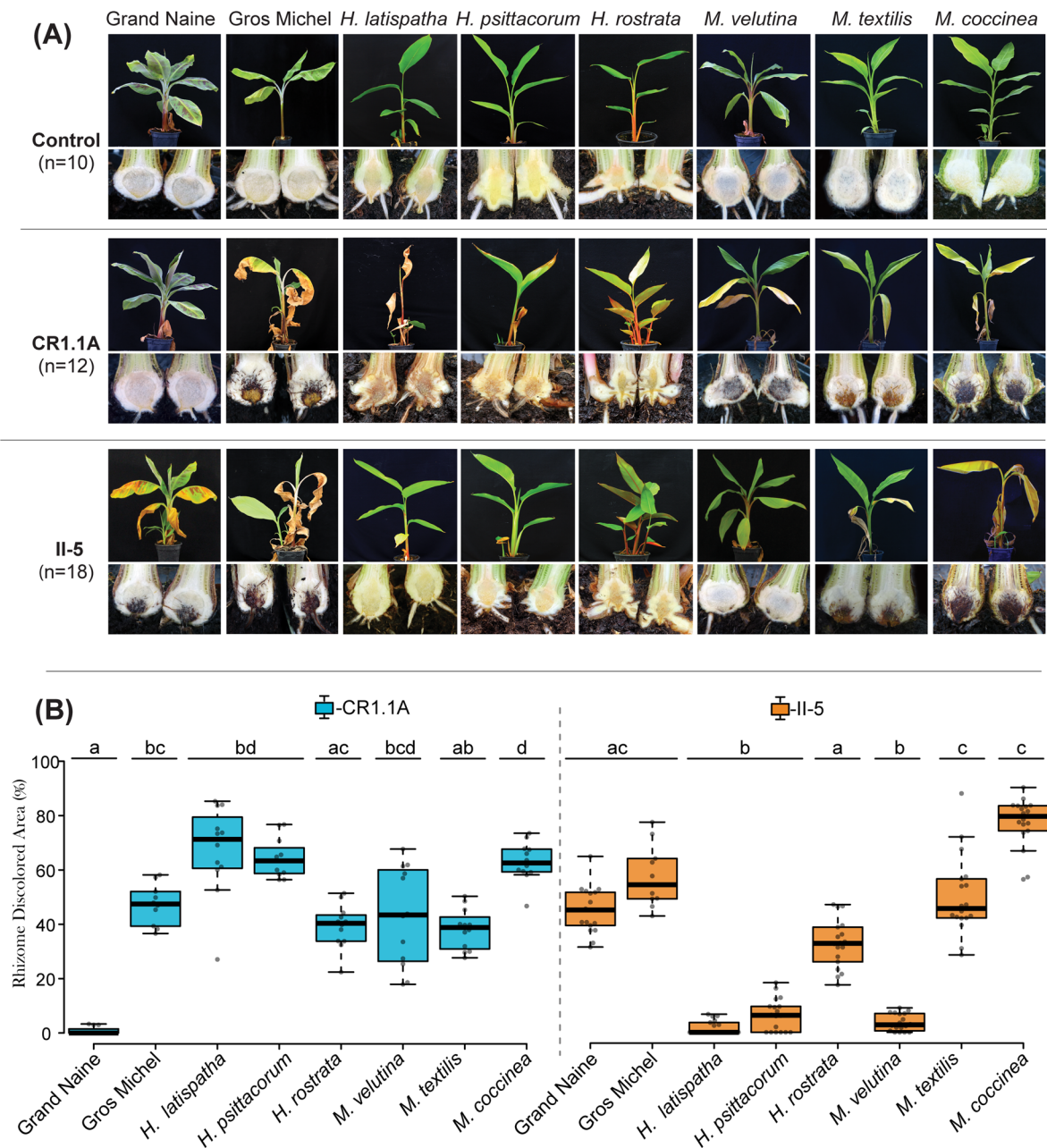


Fig. 1 Differential responses of three *Heliconia* spp. and three non-edible *Musa* spp. after inoculations with Race 1 strain CR1.1A or TR4 strain II-5. **a** External symptoms (upper rows) and cross sections of rhizomes (bottom rows) from representative plants 12 weeks after treatment with water (Control, upper panel), and inoculations with Race 1 (middle panel) or

TR4 (lower panel), n-number of replicates per treatment-plant species. **b** RDA scores of inoculated plants. Statistically significant differences ($p < 0.05$) between median RDA values are indicated with different letters, according to Dunn's multiple comparison test

inoculated *Heliconia* plants, including those of *H. rostrata*, showed necrosis in the roots and rhizomes (Fig. 1). TR4 caused rhizome discoloration in all

Heliconia species but never necrosis and only caused external symptoms in *H. latispatha*, similar to the symptoms caused by Race 1. Taken together, these

results show that the *Heliconia* spp. and *M. velutina* plants are more susceptible to Race 1 than to TR4.

To further investigate the colonization of the tested *Heliconia* spp. and *Musa* spp. by Race 1 or TR4, various parts of inoculated plants were sampled to re-isolate the *Fusarium* strains. From the total of 796 re-isolations (Table 2), representative isolates from the different plant organs per species were confirmed as Race 1 or TR4 by diagnostic PCRs (Figure S1). The presence of Race 1 or TR4 strains was confirmed in the roots, rhizomes and pseudostems of *H. latispatha*, *M. coccinea*, and *M. textilis*. We could not recover TR4 from the pseudostems of *H. psittacorum*, *H. rostrata* or *M. velutina*. Similarly, no Race 1 isolates could be recovered from the pseudostem of inoculated *H. rostrata* plants or from any tissue obtained from Grande Naine plants (Figure S1). Thus, we have demonstrated that causal agents of FWB can colonize the aboveground tissues of *H. latispatha*, *H. psittacorum*, *M. coccinea*, and *M. textilis* plants.

To determine whether the pathogenicity of the recovered isolates was altered by passage through these hosts, we used the one confirmed Race 1 and TR4 re-isolates per plant species, to inoculate Gros Michel or Grand Naine banana plants. Isolates were selected from the uppermost colonized part and their pathogenicity did not significantly differ from the TR4 and Race 1 reference strains, except the TR4 isolate recovered from *H. rostrata* which scored significantly lower than the reference strain (II-5) with an average RDA of 14.9%, (Fig. 2).

Discussion

Host plants and plant products are traded around the globe, often facilitating the unintended transport of

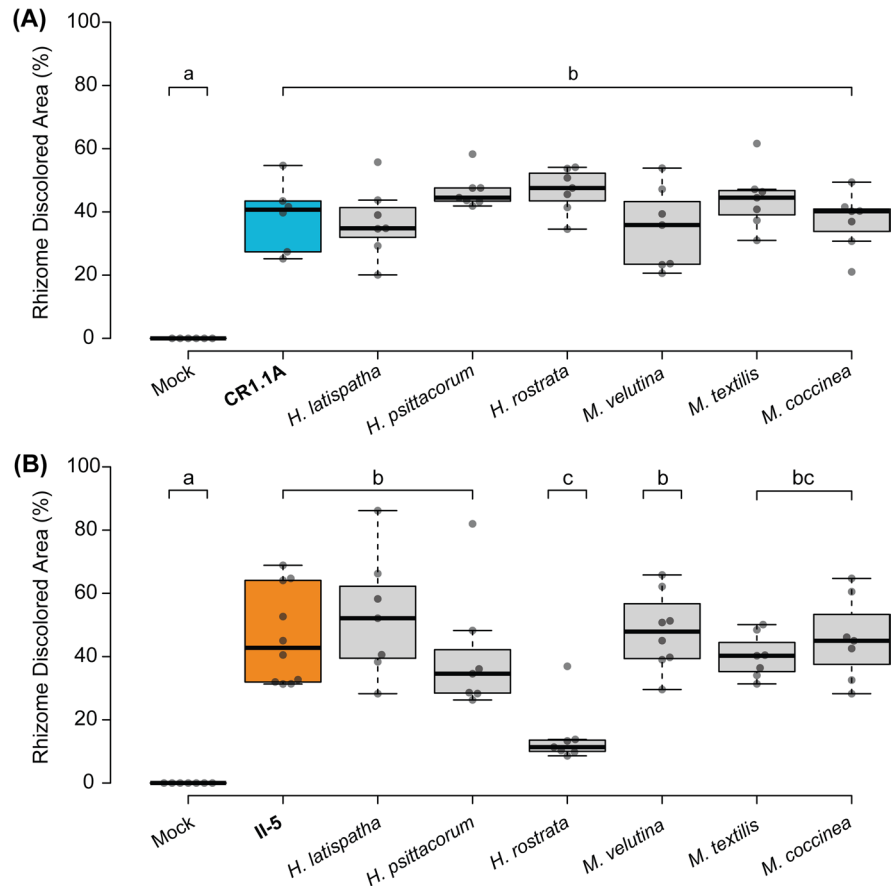
associated pathogens or endophytes (Fones et al., 2020). The increased global travel and trade over the last decades contributed to an unprecedented spread of pathogens at a global scale, with the current dissemination of TR4 as one of the clearest examples (Drenth & Kema, 2021). This worrisome global spread, and particularly the recent cases in Latin America and Mozambique (Acuña et al., 2021; García-Bastidas et al., 2020; Herrera et al., 2023; Westerhoven et al., 2022a), shows that FWB successfully disseminates despite implemented quarantine and prevention strategies (Westerhoven et al., 2022b). The recommended eradication and containment strategies, (Dita et al., 2018; Viljoen et al., 2020), largely ignore the presence of secondary hosts of the pathogen (Pegg et al., 2019). Thus, the identification of such alternative hosts could be useful in understanding pathogen persistence, inoculum accumulation, and subsequent spread to other areas (Hennessey et al., 2005).

Heliconia spp. are native to the tropical regions of the Americas, primarily Central and South America, with some species also found in the Caribbean and the Pacific islands (Gómez-Merino et al., 2018). In those regions, they are often present in rainforests, along riverbanks, and naturally growing alongside or near banana plantations. In addition, they are cultivated to produce cut flowers for the export in Latin-American and in some African countries (Linares-Gabriel et al., 2020), and are also used in landscaping parks and gardens (Gómez-Merino et al., 2018). Similar to bananas, *Fusarium* wilt is one of the most common diseases in *Heliconia* spp. (Castro et al., 2010). The causal agent of wilt in *Heliconia* spp. was initially described as Race 3 of the FWB pathogen (Waite, 1963) but is no longer considered as part

Table 2 The number of recovered isolates from different plant parts of three *Heliconia* and *Musa* species after inoculation with Race 1 and TR4

Plant species	Race 1			TR4		
	Roots	Rhizome	Pseudostem	Roots	Rhizome	Pseudostem
<i>Heliconia latispatha</i>	25	24	15	24	8	5
<i>H. psittacorum</i>	20	20	5	21	9	-
<i>H. rostrata</i>	18	6	-	14	7	-
<i>Musa coccinea</i>	31	31	22	32	30	29
<i>M. velutina</i>	35	35	30	31	21	-
<i>M. textilis</i>	31	32	17	34	28	10
Gros Michel	23	20	19	24	25	21
Grand Naine	-	-	-	34	32	30

Fig. 2 Phenotyping results of six re-isolated *Fusarium* strains from three *Heliconia* and three *Musa* spp. at 11 weeks post-inoculation. **a** Race 1 isolates tested on Gros Michel and compared with the reference strain CR1.1A. **b** TR4 isolates tested on Grand Naine and compared with the reference strain II-5. Different letters indicate statistically significant differences ($p < 0.05$) between median RDA values, according to Dunn's multiple comparison test



of the causative *Fusarium* complex infecting banana (Ploetz, 2015). Recent studies evaluating the pathogenicity of *Fusarium* strains from *Heliconia* genotypes did not include assays on bananas (Castro et al., 2008, 2010), and there is only limited information on the potential infection of *Heliconia* by FWB-causing *Fusarium* spp. This is a significant knowledge gap when considering the potential impact of global dissemination of these species, particularly since it has been shown that weeds can contribute to FWB spread which requires altered disease management strategies (Hennessy et al., 2005; Pittaway et al., 1999; Su et al., 1986; Waite & Dunlap, 1953).

Our results are mostly consistent with, but significantly extend, early studies which reported that Race 1 strains caused symptoms on *H. psittacorum* (Rishbeth, 1957), *H. caribea* and *H. latispatha* (Waite, 1961), and that re-isolated strains were pathogenic on Gros Michel. However, these re-isolated strains were obtained exclusively from the belowground plant organs (Rishbeth, 1957;

Waite, 1961), and information on the genotypes of the studied isolates is lacking. Moreover, recent phylogenetic studies confirmed that Race 1 is polyphyletic and comprises a suite of *Fusarium* lineages (Maryani et al., 2019; van Westerhoven et al. 2023). Here, we demonstrated that passing Race 1 through *H. latispatha* and *H. psittacorum* did not change the phenotype on Gros Michel. However, under our experimental conditions, *Heliconia* spp. exhibited higher susceptibility to Race 1 than to TR4. This could be attributed to the prolonged co-evolution of Race 1 with a plethora of *Heliconia* species in the Latin American and Caribbean region, which is considered the center of origin for these plants (Gómez-Merino et al., 2018; Malakar et al., 2022). We also showed for the first time that TR4 can colonize three *Heliconia* species, resulting in a range of symptoms. It is important to underscore that, in contrast to the other *Heliconia* spp., TR4 did not cause any external symptoms in *H. psittacorum* and *H. rostrata*, similar to the non-symptomatic

colonization of various weeds (Hennessy et al., 2005; Pittaway et al., 1999; Su et al., 1986; Waite & Dunlap, 1953). Taken together, our data show the flaw of the current race nomenclature, and urges adaptation of contingency and containment strategies upon the detection of TR4.

The *Musa* spp. we tested showed a varied reaction to the inoculation with the Race 1 and TR4 strains. The susceptibility to FWB of *M. textilis* was consistent with previous reports (García-Bastidas, 2019; Zuo et al., 2018), but we also observed that *M. coccinea* was equally susceptible to TR4 as to Race 1, and that *M. velutina* plants showed no wilting symptoms after inoculation with TR4, which confirms a previous report (Li et al., 2015). However, these authors neither examined the colonization of the different plant organs, nor phenotyped recovered strains from this species. We show that passage of Race 1 or TR4 through these hosts generally does not affect pathogenicity to banana, except for strains recovered from *H. rostrata*. The attenuated pathogenicity of TR4 after colonization of non-host plants and *H. rostrata* requires further studies, but the current data nevertheless underscore the need to consider the wider host range of TR4 in disease management strategies.

In conclusion, our results evidenced that non-edible *Musa* spp. and *Heliconia* spp. commonly used as fiber and ornamental crops, are vulnerable to FWB-causing *Fusarium* spp. Until more data are available, the cultivation, propagation, and trade of these species should be avoided in FWB-infested areas, as they may serve as unrecognized *Fusarium* spp. reservoirs that inadvertently contribute to FWB dissemination into new areas. Moreover, *Heliconia* spp. have also been reported as hosts of the important banana bacterial pathogen *Ralstonia solanacearum* and banana bunchy top virus, which cause Moko and bunchy top disease, respectively (Blomme et al., 2017; Hamim et al., 2017). This underscores the need for improved quarantine regulations, particularly since TR4 is geographically expanding, which threatens the sustainability of global banana production.

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