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Fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense* tropical race 4 in banana plantations in Türkiye

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Summary. Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (*Foc*TR4), is an important disease for banana production. Presence and prevalence of *Foc*TR4 in banana plantations on the Mediterranean coast of Türkiye were assessed during 2018 to 2020 in a total of 117 banana plantations in open fields and protected plastic greenhouses. Rhizome, pseudostem, and root samples were taken from plants showing typical symptoms associated to the disease and from suspected affected plants. Fungi were isolated from the plant internal tissues, and *Fusarium oxysporum*-like colonies were sub-cultured for further analyses. Phylogenetic analyses of 36 isolates showed that they belonged to four different *Fusarium* species: *F. musae, F. oxysporum, F. sacchari*, and *F. solani*. Eight representative *F. oxysporum* isolates were identified as *Foc*TR4 by specific PCR and qPCR tests. Pathogenicity tests were carried out on tissue-cultured 'Cavendish' type banana seedlings ('Grand Naine') for 36 *Fusarium* isolates, and their virulence was assessed based on the internal necrosis observed in the rhizomes. Approx. 40 to 65 d after inoculations, *Foc*TR4, *F. oxysporum*, and *F. sacchari* isolates caused severe to mild necroses in the seedling rhizomes. This is the first report of *F. sacchari* associated with root and collar rot of bananas in Türkiye. This study showed that Fusarium wilt caused by *Foc*TR4 is present, but at low incidence (6.8%) in Turkish banana plantations.

Keywords. Collar rot, Grand Naine, *Fusarium sacchari,* identification.

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INTRODUCTION

Bananas and plantains (*Musa* spp.) are important agricultural products in tropical and subtropical regions. Banana plantations are located on the coastline of the Mediterranean Region of Türkiye, and 883,455 tons of sweet bananas are produced annually from approx. 11,000 ha (Anonymous, 2022). This production does not meet the domestic consumption, and 20% of domestic consumption is imported from other banana-producing countries, including Costa Rica and Ecuador (Anonymous, 2021).

Fusarium wilt, caused by *Fusarium oxysporum* Schlecht. f. sp. *cubense* (E.F. Smith) Snyder and Hansen (*Foc*), is the most important fungal disease in banana-producing countries, due to the lack of practical management methods and the severe economic losses this disease can cause. *Foc* first infects banana plants via root hairs, then develops in the internal corm tissues, progressing to the xylem vessels and activating xylem-secreted genes that trigger initial symptoms (Dong *et al*., 2016; Czislowski *et al*., 2021). Water transport in the xylem is disrupted due to the conidial and mycelial mats produced by the pathogen, and by host substances such as gums, and mechanical barriers produced to prevent systemic spread of the fungus. Enzymes and toxins produced by *Foc* cause the host leaves to turn yellow and the lower leaves to droop (Stover, 1962).

Fusarium wilt of banana was first reported in Brisbane, Australia, in 1874 but reached severe levels in Panama and Costa Rica in the 1890s (Pegg *et al*., 2019). Destruction of the Gros Michel cultivar (susceptible to race 1 of *Foc*) by the fungus in Latin America and the Caribbean has made it one of the most important fungal pathogens wherever bananas are grown. Since the early 1960s, Fusarium wilt was not of concern due to adoption of the *Foc* resistant banana cultivar 'Cavendish'. However, despite the use of resistant 'Cavendish'-related cultivars, the disease re-appeared in the 1990s in Taiwan, Indonesia and Malaysia, caused by the new physiological race, Tropical Race 4 (*Foc*TR4) (Ploetz, 2006). To date, four physiological races of *Foc* have been described to cause Fusarium wilt, including Race 1 in 'Gros Michel' and 'Silk'; Race 2 in 'Bluggoe'; Race 3 in *Heliconia* spp., and Race 4 in all commercial banana cultivars. In addition, the subtropical race of the pathogen (*Foc*STR4) was found to be ineffective in the tropics or in areas where optimum banana growth conditions occur.

*Foc*TR4 has also caused Fusarium wilt in commercially grown banana cultivars regardless of environmental conditions (Ploetz, 2015). This race was first reported in 1990 in Taiwan; in 1992 in Indonesia and Malaysia (Ploetz and Pegg, 1997); in 1997 in Northern Australia (Conde and Pitkethley, 2001); in 1998 in China (Qi *et al*., 2008); in 2005 in the Philippines; in 2012 in the Sultanate of Oman; in 2013 in Mozambique and Jordan (García-Bastidas *et al*., 2014; Perez-Vicente *et al*., 2014; Viljoen *et al*., 2020); in 2015 in Lebanon and Pakistan (Ordonez *et al*., 2016); in 2018 in Vietnam (Hung *et al*., 2018); in Laos (Chittarath *et al*., 2018), Myanmar (Zheng *et al*., 2018) and Israel (Maymon *et al.,* 2018); in 2019 in India (Thangavelu *et al.,* 2019), Thailand and Colombia (García-Bastidas *et al*., 2020); in 2020 in Türkiye (Özarslandan and Akgül, 2020); in 2021 in Mayotte (Aguayo *et al*., 2021), and Peru (Acuna *et al*., 2022); and in 2022 in Venezuela (Mejias Herrera *et al*., 2023).

As with other soil-borne plant pathogenic fungi, managing *Foc*TR4 is challenging, and practical control has not yet been found for eradicating the pathogen from soil. *Foc* has long persistence in the soil (more than 20 years), due to its ability to survive as a saprophyte in plant residues and to produce chlamydospores. As these inocula are pushed deeper into the soil with tillage, eliminating the pathogen from infested areas becomes almost impossible (Stover, 1962). Solarization is ineffective in eradicating this pathogen when applied alone, because of its inability to penetrate deep into the soil (Herbert and Marx, 1990). In addition, using this method in large areas is difficult and costly. Breeding for resistance is ongoing, but a banana cultivar with high commercial potential and complete resistance has not yet been produced, as was previously the case for the *Foc*1 and *Foc*2 resistant 'Cavendish' (Dita *et al*., 2018).

Fusarium oxysporum is a large species complex including saprophytes, endophytes, plant and human pathogens; it also has 143 *formae speciales* (106 of them are well-documented) and 25 physiological races (including *Foc*TR4) from monocotyledon and dicotyledon host plants (Edel-Hermann and Lecomte, 2019). Formerly, *Fusarium* species have been identified based on their morphological characteristics, which primarily include asexual reproductive structures (chlamydospores, conidiophores, macro- and microconidia, and phialides) and colony morphology (Fourie *et al.,* 2009). However, molecular tools and phylogenetic analyses supported by DNA gene sequencing, as well as pathogenicity tests, have shown that these features are insufficient for species identification. Since it is not possible to discriminate pathogenic isolates (or physiological races) using morphology and microscopy, precise diagnostic procedures (e.g. PCR, gene sequencing, VCG tests) and pathogenicity tests must be used for accurate identification. In the last 15 years, commercial diagnostic kits and PCR primers that amplify specific regions in genomic DNA of these races have been designed for molecular identifications. Lin *et al.* (2009) followed the RAPD marker technique to design specific primers for identifying tropical race 4 using 96 *Foc*TR4 isolates from Taiwan. Their primer (*Foc*1/*Foc*2) amplifies a specific 242-bp gene product in *Foc* genomic DNA. Dita *et al.* (2010) designed *Foc*TR4-F/*Foc*TR4-R primers to identify *Foc*TR4 strains using single nucleotide polymorphism in the IGS region. These primers have been widely used for monitoring the disease and in pathogen identification studies. Some commercial diagnostic kits containing pathogen-positive DNA have also been developed for qPCR ampli-

fication, and have been used in studies aimed at disease monitoring in banana-producing countries (Dale *et al*., 2017; García-Bastidas *et al.,* 2019).

If the pathogen has not appeared in a country, the most effective control measure is to exclude the pathogen by implementing strict quarantine measures. When the pathogen is detected in a limited area, it is necessary to contain and eradicate it before becoming widespread. Pathogen tracing and disease monitoring with regular surveys, and accurate identification procedures, are essential. Fusarium wilt of banana was first reported in Türkiye was in 2018. *Foc*TR4 was detected in three protected plastic covered greenhouses after identification using specific PCR primers and pathogenicity tests (Özarslandan and Akgül, 2020). However, a more comprehensive study is required to determine prevalence of Fusarium wilt in Türkiye.

The aim of the present study was to determine the prevalence of Fusarium wilt caused by *Foc*TR4 in open-field and plastic-covered-greenhouse banana plantations in Türkiye. Knowledge of *Foc*TR4 prevalence in these areas would provide a basis for appropriate quarantine measurements, and for further investigations on disease management and screening of local banana cultivars for resistance breeding.

MATERIALS AND METHODS

Survey, sample collection and isolation of fungi

A survey was carried out in protected plastic, greenhouses (n = 72) and open field banana plantations (n = 45) located in Seyhan, Yüreğir (Adana province), Alanya, Gazipaşa (Antalya province), Arsuz (Hatay), and Anamur, Bozyazı, Erdemli, Silifke, Tarsus districts (Mersin province), in the Mediterranean Region of Türkiye (Figure 1). A total of 117 plantations (approx. total area 76 ha) were inspected, from March 2018 to December 2020. Individual banana the plants were examined for general appearance, and rhizome, pseudostem, and root samples were collected from those with typical disease symptoms (Figure 2). These samples were placed in paper bags and then in an ice box, and transported to a laboratory for further processing.

Small sections (5 to 8 cm each) of symptomatic pseudostem, corm, and root tissues were dissected from each sample, and then surface sterilized with 2.5% sodium hypochlorite solution (>5% active chlorine) for 3 min, then rinsed twice in sterile distilled water. The internal tissues (4 to 6 mm) from each section were aseptically placed onto ¼ strength Potato Dextrose Agar (PDA; CondaLab) amended with streptomycin sulfate (250 µg mL^{-1}), and the culture were incubated at 25°C for 4-5 d. Twenty Petri dishes were used isolations from tissues from each banana plantation. After morphological and microscopic examinations, *Fusarium oxysporum*-like colonies were transferred by single-conidium isolation techniques onto fresh PDA plates, backed up on sterile green banana leaf cultures, and were stored at -20°C (Seifert, 1996). Among the *F. oxysporum*-like fungi, a representative sample of 36 isolates was selected for further pathogenicity testing and gene sequencing. These isolates were tentatively identified using cultural and microscopical characteristics (fluffy mycelia, simple short phialides, and pale violet or pinkish colony colour), as described by Nelson (1983) and Leslie and Summerell (2006). *Foc*TR4 prevalence (%) was calculat-

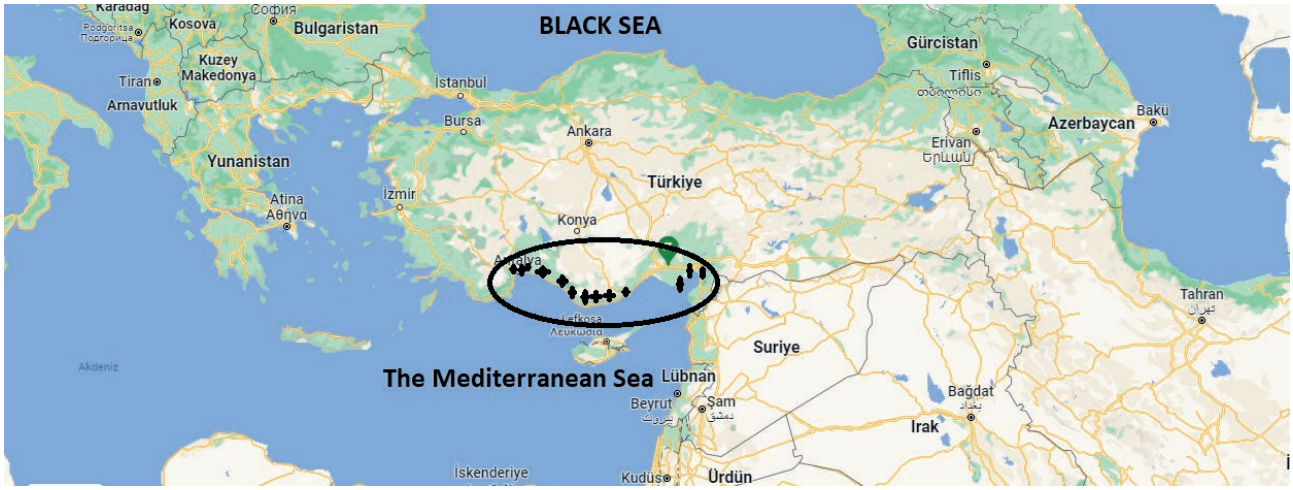

Figure 1. The location of surveyed banana plantations in the Mediterranean Region of Türkiye.

Figure 2. A protected banana greenhouse with wilt symptomatic plants, and vascular necrosis in an affected pseudostem.

ed based on the number of greenhouse or banana fields (where *Foc*TR4 detected and the total number of banana plantations (surveyed). The isolation frequency (%) of all fungi in each plantation was estimated using 140 tissue pieces plated (20 Petri plates, seven tissue fragments each) from symptomatic plants.

Molecular identification of Fusarium *isolates*

Thirty-six *F. oxysporum*-like isolates (tentatively identified based on their simple short phialides on hyphae, salmon or pale violet colony colour after 3 weeks incubation on PDA at 24°C in the dark) were selected for further molecular identification. Isolates were grown on PDA at 24°C for 8-10 d in the dark. Fresh aerial mycelia were collected by scraping the colonies with a sterile scalpel, and the mycelia were placed in sterile Eppendorf tubes containing 2% CTAB buffer. The following extraction steps were then followed, as described by O'Donnell *et al*. (1998). For gene sequencing, translation elongation factor 1α (TEF-1α) and intergenic spacer (IGS) region (nuclear ribosomal operon) genes were amplified using PCR using, respectively, the EF1/EF2 primer (O'Donnell *et al*., 1998) and *Foc*TR4F/*Foc*TR4R primer (Dita *et al*., 2010). Each PCR reaction mixture contained 5 µL of buffer (10× Green Buffer, DreamTaq Green DNA Polymerase, Thermo Scientific™), 2 µL of the dNTPs mixture (10 mM each, Thermo Scientific™), 0.5 µL of forward and reverse primers (stock concentration: 10 pmol·µL^{-1}), 0.25 µL of Taq polymerase (DreamTaq Green DNA Polymerase, Thermo Scientific™), 39.75 µL of PCR grade water and 1 µL of genomic DNA (approx. 100 ng·µL^{-1}). PCR amplifications were carried out in a thermocycler (Simpli-Amp A24811™ Thermal Cycler, Applied Biosystems), using the conditions specified in Table 1. PCR products were separated by gel electrophoresis in 1.5% agarose

Table 1. PCR conditions used in this study to amplify partial genomic DNA of *Fusarium* isolates.

Amplification stages	EF1/EF2			*Foc*TR4/*Foc*TR4R			Clear Detections Kit™		
	Temp. (°C)	Duration (min)	No. of cycles	Temp. (°C)	Duration (min)	No. of cycles	Temp. (°C)	Duration (sec)	No. of cycles
Initial denaturation	95	3	1	95	5	1	95	3	1
Denaturation	95	1	35	95	1	30	95	10	40
Annealing	52	1		60	1		63	60	
Extension	72	1.5		72	3		72	30	
Final extension	72	10	1	72	10	1	70	60	1

(Sigma) gels in 1× Tris-Acetic acid-EDTA (TAE) buffer, to check DNA band size and quality. These products were then sequenced by Macrogen Co. (South Korea), and the sequences were compared with those deposited in the NCBI GenBank database using the BLAST tool (version 2.0; National Center for Biotechnology Information, US National Institutes of Health). TEF-1α and IGS sequences obtained were deposited to the NCBI GenBank.

FocTR4 identification was also confirmed by Real-Time PCR using SYBR® Green technology (Clear Detections TR4 Kit™). For each isolate, 5 µL of genomic DNA was added to 200 µL capacity thermocycler tubes each containing 15 µL of Clear Detections qPCR master mix, then vortexed for 20 sec. of PCR-grade water (5 µL) and *Foc*TR4 genomic DNA (provided by the manufacturer) were then added to other PCR tubes to confirm negative and positive amplifications as controls. The thermal cycler (Roche Light Cycler 480™) was set according to the conditions outlined in Table 1, and whether the isolates were *Foc*TR4 was determined according to the obtained cycle thresholds (Ct). To avoid missing weak positive amplifications due to DNA quality and other factors, melting-curve analysis was also carried out, and amplifications around 75°C were considered primer dimers. The molecular identification tests were each repeated once.

The phylogenetic analysis was carried out using data from the translation elongation factor-1α gene. The data set was constructed using reference sequences (Table 2) from relevant publications (Maryani *et al*., 2019a; Crous *et al*., 2021; Tava *et al*., 2021). The sequences were aligned using the algorithm in Muscle, and a maximum likelihood tree was constructed using MEGA-X software with the Hasegawa-Kishino-Yano model (Hasegawa *et al*., 1985). *Geejayessia zealandica* (= *F. zealandicum*) CBS isolate 111.93 was used as a root taxon, and node support was estimated by bootstrap analysis on 1000 random trees.

Table 2. GenBank accession numbers of partial sequence of TEF 1-α of reference species used in the phylogenetic analyses.

Species	Isolate	GenBank Accession No.
Fusarium grosmichelii	InaCC F820	LS479810
Fusarium musae	F31	MW916961
Fusarium oxysporum	CAV794	FJ664922
Fusarium oxysporum	CAV189	FJ664956
Fusarium oxysporum	NRRL26029	AF008493
Fusarium oxysporum f. sp. *cubense* TR4	FocII5 NRRL36104	LS479644
Fusarium oxysporum f. sp. *cubense* TR4	InaCC F816	LS479677
Fusarium *oxysporum* f. sp. cubense TR4	InaCC F817	LS479753
Fusarium philaophorum	FocIndo25	LS479650
Fusarium proliferatum	NRRL62905	KU171727
Fusarium purpurascens	InaCC F823	LS479838
Fusarium sacchari	NRRL13999	AF160278
Fusarium solani	CBS 102429	KM231936
Fusarium solani	KARE233	MK077039
Fusarium tardichlamydosporum	FocCNPMF-R2	LS479643

Pathogenicity tests

The 36 selected *Fusarium* isolates were used in pathogenicity tests on banana seedlings, as described by Thangavelu *et al*. (2019). The isolates were grown on PDA for 7-8 d at 25°C in the dark. The mature cultures were then flooded with sterile distilled water, and mycelia were scraped with a sterile plastic needle to dislodge macro and microconidia. The conidium suspensions were each filtered through two layers of sterilized cheesecloth, and the concentration of resulting conidium suspension was adjusted to 10^6 conidia mL^{-1}, after enumeration with a Thoma® slide under a light microscope. Banana seedlings ('Grand Naine' at 4 to 6 leaf stage, produced by tissue culture), were uprooted

from trays; their roots were slightly trimmed and then dipped into the conidium suspensions of respective isolates for 10 min. The seedlings were then planted in plastic pots (15 cm diam.) containing sterile peat moss, sand and perlite mixture (1:1:1 v/v/v), and were placed in climate controlled greenhouses (at 27°C, 85% relative humidity, 12 h illumination). The inoculated plants were grown for 65 d, and the pathogenicity of the isolates was assessed using a 0 to 3 severity scale, based on the discolouration of the each plant rhizome and whole plant wilting, as described by Li *et al*. (2015). Disease severity was calculated using the formula of Towsend and Heuberger (1943): (∑ (number of plants in a disease scale category × disease scale category) / (total number of plants × maximum disease scale category)) ×100). Eight plants (one plant per pot and four replicates with two plants per replicate) were inoculated with each *Fusarium* isolate. Inoculation control plants were treated with sterile distilled water. Pathogenicity was confirmed by re-isolating inoculated isolates from roots and necrotic internal tissues of plants. The pathogenicity tests were each repeated once.

Statistical analyses

Analysis of variance (ANOVA) were carried out on disease severity data (mean lesion lengths in two experiments), and the data were checked for normality. Means were compared using Fisher's least significant difference (LSD) test at $P \leq 0.05$ (Gomez and Gomez, 1984).

RESULTS

Fungal isolations, identification of Foc*TR4, and disease severity assessments*

In the surveyed banana plantations, 12 fungal genera, including *Alternaria, Aspergillus, Cladosporium, Epicoccum, Fusarium, Macrophomina, Nigrospora, Penicillium, Pythium, Phytophthora, Rhizoctonia,* and *Trichoderma* (based on ITS sequencing and simple BLAST searches), were obtained from symptomatic and asymptomatic banana plants. Among these fungi, *Fusarium* was the most commonly isolated genus, obtained from 96% (112 of 117 plantations) of the total plantations assessed. According to cultural morphological characteristics of isolates (fluffy mycelia, simple short phialides, and pale violet or pinkish colonies) and microscopic features (short or long simple phialides, macro- and microconidium shapes, chlamydospore production), 36 *Fusarium* isolates were selected for further identification studies.

A nucleotide BLAST search using the translation elongation factor 1-α gene revealed four different *Fusarium* species; *F. musae*, *F. oxysporum*, *F. sacchari*, and *F. solani* (Table 3). Of the 36 selected *Fusarium* isolates, most belonged to *F. oxysporum* (25 isolates), followed by *F. sacchari* (eight isolates), *F. solani* (two isolates), and *F. musae* (one isolate).

The BLAST results were confirmed by the clustering of the isolates with respective reference sequences of *F. musae* (F31), *F. oxysporum* (CAV794, NRRL_26029), *F. sacchari* (NRRL_13999), and *F. solani* (CBS 102429) in the phylogenetic tree. However, these methods could not differentiate *Foc*TR4 from the closely related *Fusarium oxysporum* isolates.

The conventional PCR tests showed that eight of the *F. oxysporum* isolates belonged to the tropical race four, so 463 bp DNA bands were observed with agarose gel electrophoresis (Figure 4). However, genomic DNAs from the remaining 28 isolates could not be amplified using these specific primers.

The real-time PCR tests using a *Foc*TR4-specific diagnostic kit agreed with the conventional PCR results. On average, while the CT value average for the reference DNA provided by the Clear Detection™ commercial kit was 21.59, these values varied between 24.97 and 31.63 in eight isolates suspected to be *Foc*TR4 (Figure 5). No amplification was recorded for DNA of the other *Fusarium* isolates (including the water control), and their Ct values were greater than 36 (Table 3).

Pathogenicity tests

Approx. 40 d after inoculating 'Grand Naine' banana seedlings with the *Fusarium* isolates, some plants showed yellowing of the lower leaves (Figure 6, a and b). Plants inoculated with two *Fusarium solani* isolates (BMAE41 and BMAE43), *F. musae* (BMAE3MM), or the non-inoculated control plants, did not develop disease symptoms (Figure 6 j). Approx. 50 d post inoculation, yellowing symptoms progressed to the upper leaves, while the lower leaves wilted and dried thoroughly. Two weeks after these symptoms appeared, plants inoculated with isolates identified as *Foc*TR4 wilted and died, while plants inoculated with the other isolates continued to live for approx. 20 d. Rhizome necroses started from the pith tissues in *Foc*TR4 inoculated plants (Figure 6 c to f), this progressed from the cortex to the centres in *F. oxysporum* and *F. sacchari* inoculated plants (Figure 6 g and h). According to the evaluation scale of

Table 3. *Fusarium* species, isolate identification numbers, source banana plantation locations, and cultivars, isolate translation elongation factor (TEF 1-α) and 28S-18S intergenic spacer (IGS) GenBank accession numbers, and cycle threshold (Ct) values from Real-Rime PCR.

Fungal Species	Isolate	Location	Cultivar	GenBank Accession Numbers		Ct Values at
				TEF 1-α	IGS	Real_Time PCR
Fusarium musae	BMAE3MM	Erdemli, Mersin	Grand Naine	OM350374	-	NA
Fusarium sacchari	BMAE4MM	Erdemli, Mersin	Azman	OM350339	-	"
	BMAE5MM	Erdemli, Mersin	Azman	OM350340	-	"
	BMAE8MM	Silifke, Mersin	Grand Naine	OM350375	-	"
	BMAE11MM	Silifke, Mersin	Grand Naine	OM350343	-	"
	BMAE44MM	Bozyazı, Mersin	Azman	OM350348	-	"
	BMAE101MM	Anamur, Mersin	Grand Naine	OM350379	-	"
	BMAE103MM	Bozyazı, Mersin	Grand Naine	OM350364	-	"
	BMAE107MM	Arsuz, Hatay	Grand Naine	OM350368	-	"
F. oxysporum f. sp. *cubense* TR4	BMAE9MM	Silifke, Mersin	Grand Naine	OM350342	OM350369	27.92
	BMAE36MM	Anamur, Mersin	Grand Naine	OM350345	OM350370	24.97
	BMAE49MM	Bozyazı, Mersin	Bodur Azman	OM350350	OM350371	26.88
	BMAE70MM	Gazipaşa, Antalya	Bodur Cavendish	OM350354	MN419031	28.92
	BMAE83MM	Alanya, Antalya	Bodur Cavendish	OM350356	OM350372	31.63
	BMAE87MM	Alanya, Antalya	Bodur Cavendish	OM350357	MN419032	26.73
	BMAE102MM	Anamur, Mersin	Bodur Azman	OM350363	OM350373	26.30
	BMAE104MM	Anamur, Mersin	Azman	OM350365	MN419033	26.00
F. oxysporum	BMAE7MM	Silifke, Mersin	Grand Naine	OM350341	-	NA
	BMAE20MM	Arsuz, Hatay	Grand Naine	OM350376	-	"
	BMAE35MM	Anamur, Mersin	Bodur Azman	OM350344	-	"
	BMAE46MM	Bozyazı, Mersin	Şimşek	OM350349	-	"
	BMAE61MM	Alanya, Antalya	Bodur Cavendish	OM350351	-	"
	BMAE62MM	Alanya, Antalya	Bodur Cavendish	OM350377	-	"
	BMAE63MM	Alanya, Antalya	Bodur Cavendish	OM350352	-	"
	BMAE69MM	Alanya, Antalya	Bodur Cavendish	OM350353	-	"
	BMAE79MM	Alanya, Antalya	Bodur Cavendish	OM350355	-	"
	BMAE93MM	Alanya, Antalya	Bodur Cavendish	OM350358	-	"
	BMAE96MM	Alanya, Antalya	Bodur Cavendish	OM350359	-	"
	BMAE97MM	Alanya, Antalya	Bodur Cavendish	OM350360	-	"
	BMAE98MM	Alanya, Antalya	Bodur Cavendish	OM350361	-	"
	BMAE99MM	Alanya, Antalya	Bodur Cavendish	OM350362	-	"
	BMAE100MM	Alanya, Antalya	Bodur Cavendish	OM350378	-	"
	BMAE105MM	Silifke, Mersin	Grand Naine	OM350366	-	"
	BMAE106MM	Silifke, Mersin	Grand Naine	OM350367	-	"
F. solani	BMAE41MM	Bozyazı, Mersin	Azman	OM350346	-	"
	BMAE43MM	Bozyazı, Mersin	Azman	OM350347	-	"

Li *et al*., (2015), *F. sacchari* and *F. oxysporum* caused rhizome necrosis and seedling death at rates from 33.3% to 83.3%, while no symptoms were observed from *F. solani* (BMAE41MM and BMAE43MM) or *F. musae* (BMAE-3MM) isolates and the sterile water inoculated controls (Figure 6 j). Disease severity was greater (66.7-91.7%) in plants inoculated with *Foc*TR4 isolates (Figure 7). *Fusarium* isolates were re-isolated from internal rhizome tissues of these plants. *Fusarium solani* isolates were re-isolated only from the hairy roots (not rhizomes), while no *Fusarium* colonies were obtained from the rhizomes of the non-inoculated control plants. Based on the overall averages of all isolates from each species, *F. oxysporum* f. sp. *cubense* TR4 caused the most severe rhizome necrosis (74.0%), followed by *F. sacchari* (61.5%) and *F. oxysporum* (58.3%).

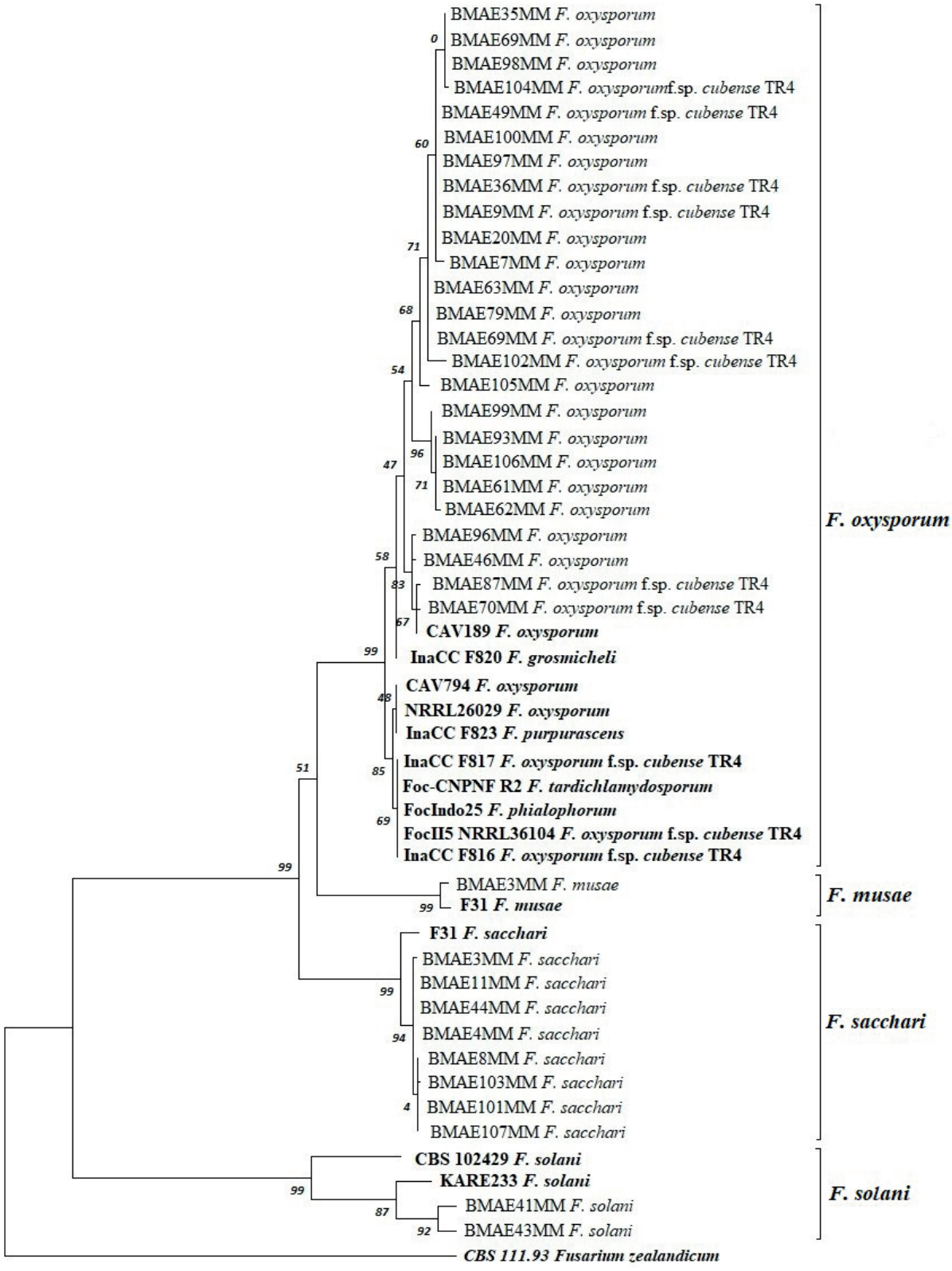

Figure 3. Maximum likelihood analysis of *Fusarium* species isolates, based on TEF-1α gene sequences. Numbers above the branch nodes represent bootstrap values from 1,000 replications. The sequence of the TEF-1α gene from *F. zealandicum* isolate CBS 111.93 was the outgroup used to root the tree.

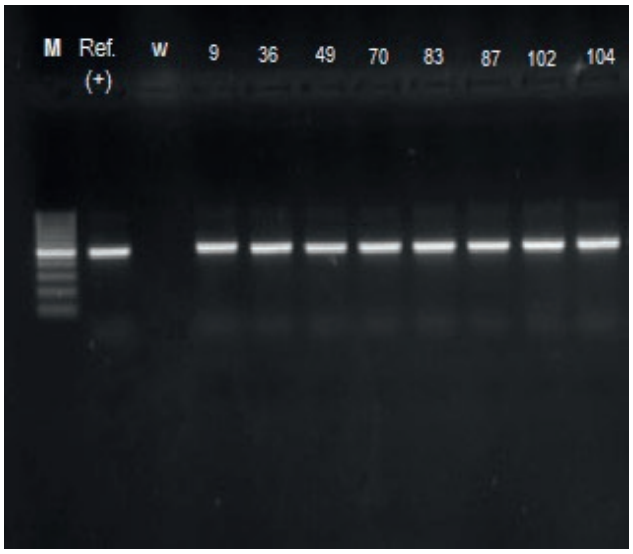

Figure 4. Electrophoretic separation of DNA bands (463 bp) of *Foc*TR4 isolates obtained from *Foc*TR4F/*Foc*TR4R primer pairs. Lane M, DNA ladder (Thermo Scientific); lane 1, Reference *Foc*-TR4 (Jordan); lane 2: PCR-grade water; lane 3, BMAE9MM; lane 4, BMAE36MM; lane 5, BMAE49MM;, lane 6, BMAE70MM; lane 7, BMAE83MM; lane 8, BMAE87MM; lane 9, BMAE102MM; lane 10,: BMAE104MM.

DISCUSSION

Fusarium wilt of banana is a severe disease affecting banana plantations, and is ranked among the top ten most important fungal diseases (Dean *et al*., 2012). The physiological races of *F. oxysporum* f. sp. *cubense* were unable to infect resistant banana cultivars until the 1990s, but the new race (Tropical Race 4) overcame this resistance in 1992 in Southeast Asia, and rapidly spread across banana producing areas. *Foc*TR4 has since been reported in more than 20 countries (Bregard *et al.,* 2022). The present study detected *Foc*TR4 in eight of 117 plantations (6.8%) in Türkiye, and all eight were in protected plastic greenhouses. No plants showing typical disease symptoms were found in open field plantations, and the pathogen was not detected in suspected plants. The limited detection of the pathogen in Türkiye is probably because bananas are mostly grown in protected greenhouses, with limited access and under drip irrigation systems, limiting the spread and proliferation of the pathogen. Growing conditions in other banana-growing countries are different, with most banana plantations located in open fields exposed to tropical climates (high precipitation and warm tem-

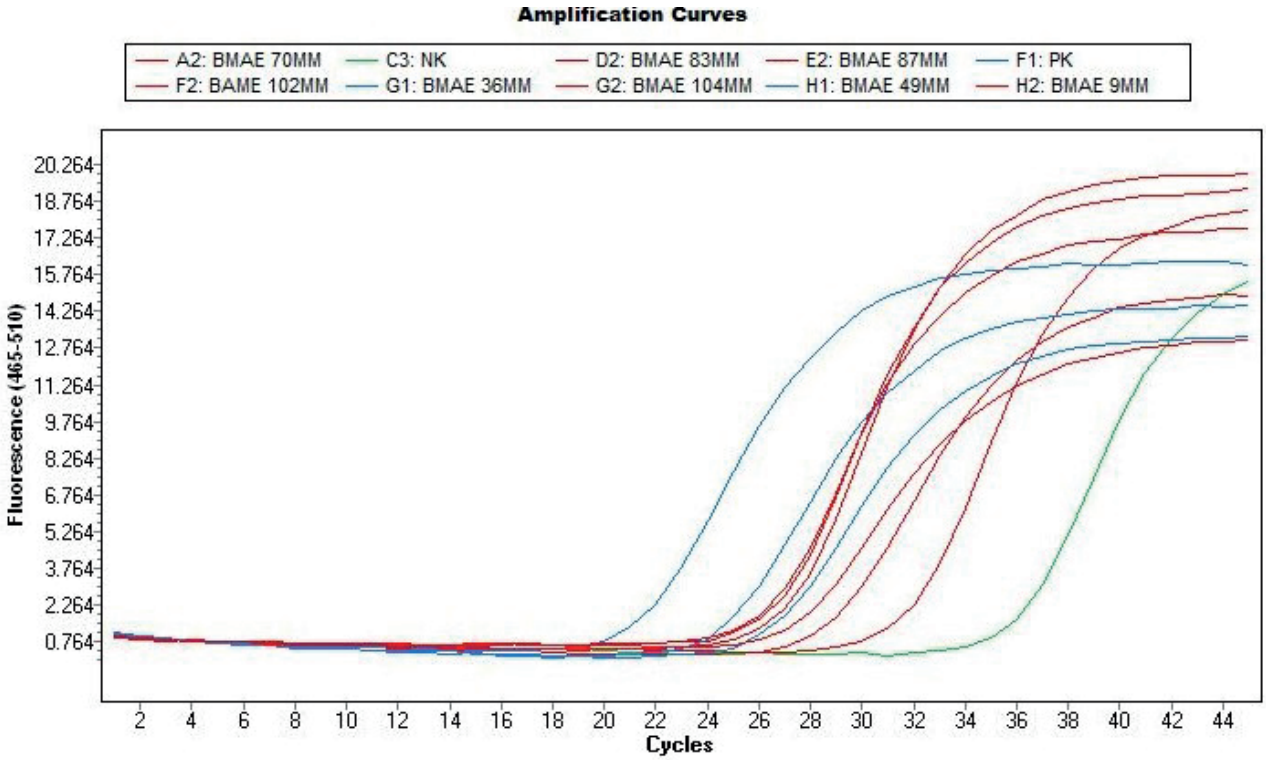

Figure 5. Real-time PCR amplification curves for *Foc*TR4 isolates (curves A2, D2, E2, F2, G1, G2, H1, and H2), and the reference positive control (curve F1) and water control (curve C3).

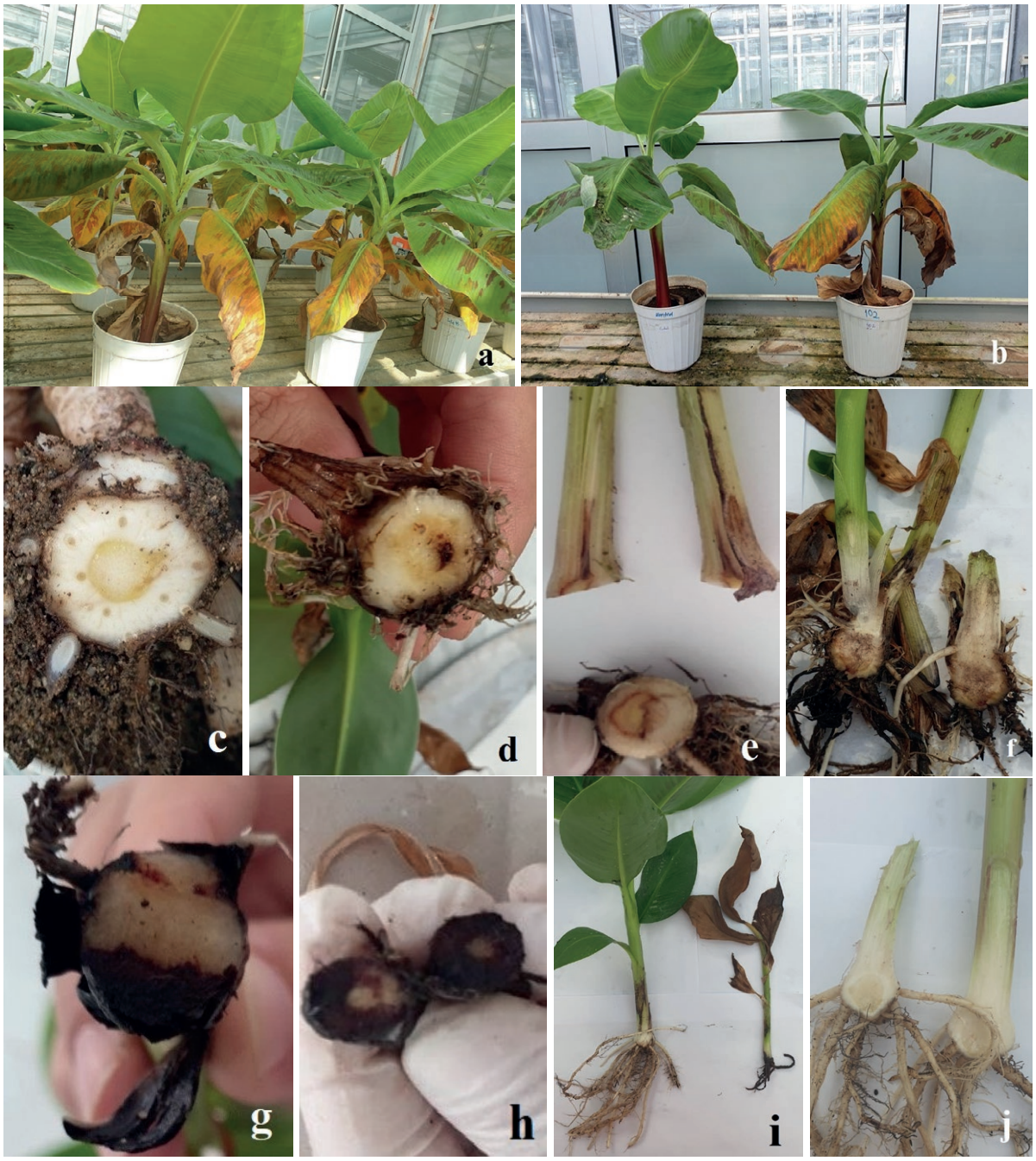

Figure 6. Leaf yellowing (a), wilt (b and i), and rhizome necrosis, caused by isolates of *Fusarium oxysporum* f. sp. *cubense* TR4 (c to f), *F. oxysporum* (g) and *F. sacchari* (h), and non-inoculated controls or *F. musae* or *F. solani* inoculated plants (j), 65 d after inoculations in greenhouse conditions.

peratures). This is probably why the disease is more prevalent in other countries.

Karangwa *et al.* (2016) assessed the distribution and incidence of Fusarium wilt in Burundi, the Democratic Republic of Congo, Rwanda, and Tanzania. They reported that 54.1% of the banana plantations had disease incidences greater than 40%, and the greatest incidence (63.6%) was in Tanzania. Zheng *et al.* (2018) surveyed banana fields in Laos, Myanmar, Vietnam, and Yunnan Province of China, to determine presence of *Foc*TR4. *Fusarium oxysporum*-like isolates were recovered from symptomatic plants from 25 banana fields, and 81.3% of

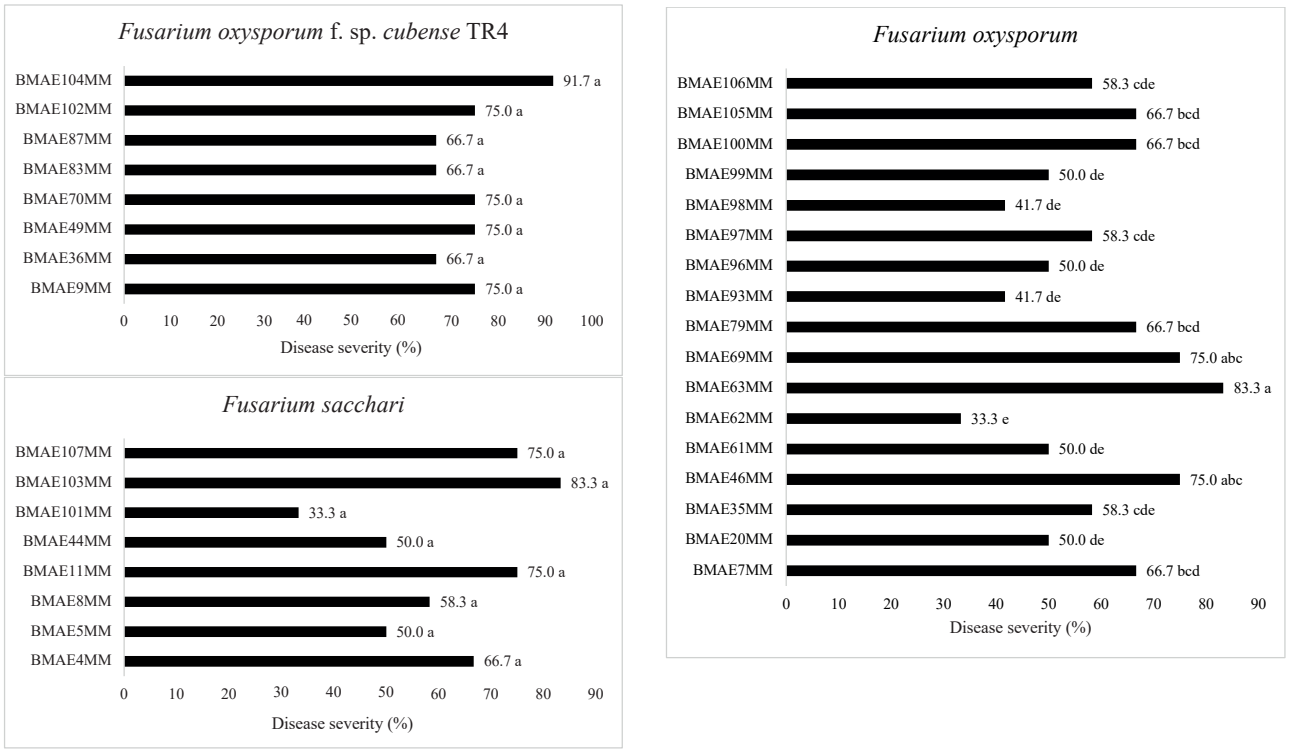

Figure 7. Mean disease severity percentages in corm tissues of 'Grand Naine' banana seedlings (inoculated at the 4 to 6 leaf stage) caused by the *Fusarium oxysporum* f. sp. *cubense* TR4, *F. sacchari* and *F. oxysporum* isolates in greenhouse conditions.

these were identified as *Foc*TR4. In a survey by Thi *et al.* (2022) in Vietnam, Fusarium wilt associated with *Foc*-TR4 was not widespread in Vietnam. Using molecular tools, these authors analyzed 19 *Fusarium* isolates from three different geographical regions, and found that only 10% were *Foc*TR4.

In the present study, *Fusarium* isolates were obtained from banana roots, rhizomes, and internal pseudostem tissues, and this was the most frequently isolated genus (96% of isolates). In addition to *Fusarium*, soilborne phytopathogenic fungi and fungus-like organisms (including *Macrophomina, Pythium, Phytophthora,* and *Rhizoctonia*) associated with root and rhizome rot, and common endophytes of bananas (*Alternaria, Aspergillus, Cladosporium, Epicoccum, Nigrospora, Penicillium, Trichoderma*), were also isolated from these tissues. More than 100 *Fusarium* isolates were obtained, from which 36 that were morphologically similar to *Fusarium oxysporum* were selected and taken to the next stages of identification. Phylogenetic analysis showed that nine of these isolates were in the *F. fujikuroi* species complex, 25 were in *F. oxysporum*, and two in the *F. solani* species complex. Among these complexes, four different *Fusarium* species (*F. musae, F. oxysporum, F. sacchari,* and *F. solani*) were identified.

Maryani *et al.* (2019a) obtained many *Fusarium* isolates from 34 geographically and environmentally different locations in Indonesia, and identified 200 of them using detailed phylogenetic analyses. The pathogen community included 14 *Fusarium* species within four species complexes, and 180 isolates were within *F. oxysporum* species complex (FOSC). Ujat *et al.* (2021) studied *Fusarium* species diversity in 17 regions of Malaysia, and found that most of the 38 isolates (86.8%) obtained were in the FOSC. Within this complex, *F. oxysporum* f. sp. *cubense* was the most frequently isolated species (71.1%), followed by *F. oxysporum* (10.5%) and *F. grosmicheli* (5.3%). Similarly, Karangwa *et al.,* (2018) discriminated *Fusarium* isolates obtained from East and Central African countries using phylogenetic analysis and VCG tests, and identified *F. sacchari* and *F. semitectum* as well as *Fusarium oxysporum* f. sp. *cubense*. Although some of these *F. oxysporum* isolates were confirmed to be pathogenic to banana seedlings, these isolates could not be discriminated using VCG tests. Czislowski *et al.* (2021) obtained 105 isolates from plantations in Australia, from symptomatic and asymptomatic banana plants, recovering fungi belonging to the *F. fujikuroi* and *F. oxysporum* species complexes more frequently than those of *F. solani and F. incarnatum-equiseti* complexes. *Fusarium*

solani and *F. fujikuroi* were found to predominate in the other species complexes. Results from these studies indicate that Fusarium wilt may have different prevalence in different countries, and that banana decline can be caused by non-*Foc*TR4 species.

The TEF 1-α gene was amplified by PCR and sequenced in the present study to make robust distinction between morphologically similar *Fusarium* isolates. While this approach identified species within species complexes, it was insufficient to distinguish between *formae speciales* and physiological races within the *F. oxysporum* species complex. To make robust phylogenetic discrimination between *Fusarium* species, O'Donnell *et al.* (2022) suggested that the RPB1 or RPB2 genes should be studied together with the TEF 1-α gene, but noted that TEF 1-α sequencing results would also be valuable in cases of limited resources. Ujat *et al.* (2021) performed phylogenetic differentiation of *Fusarium* isolates in bananas by sequencing the TEF or Histone-H3 genes, and suggested that working with the TEF 1-α gene revealed more consistent results than those from Histone-H3. Maryani *et al.* (2019a) carried out phylogenetic analyses by combining RPB1, RPB2, and TEF 1-α genes, and made detailed identification of *Fusarium* species complexes from bananas and the species in these complexes. Greatest phylogenetic support was obtained in their study by combining RPB1 and TEF 1 genes. Phylogenetic analyses using the sequences of gene regions proposed by Maryani *et al.* (2019a) could be used to identify *F. oxysporum* f. sp. *cubense*, and assess relationships among isolates. However, since the presence of *Foc*TR4 was to be investigated in the present study by other methods (*Foc*TR4-specific primers and a commercial identification kit), phylogenetic analyses with only the TEF 1-α gene was used as a first stage to confirm sequencing results.

The *Foc*TR4-specific primers (*Foc*TR4F/*Foc*TR4R) identified eight out of the 36 selected isolates, and results were validated with a commercially available diagnostic real-time PCR kit (Clear Detections™) designed for *Foc*TR4 detection. These two detection methods were used to detect first incursions of the pathogen in Colombia (in 2020) and Peru (in 2022). García-Bastidas *et al.* (2020) first tested the *Fusarium* isolates with *Foc*TR4-specific primers to detect the presence of *Foc*TR4 in banana plantations in Colombia. They also confirmed the presence of *Foc*TR4 using the Clear Detection Kit™, loop-mediated isothermal amplification, and whole genome analyses. Acuna *et al.* (2022) examined *Fusarium* isolates from a suspected *Foc*TR4 infested banana plantation in Querecotillo, Peru, and detected this race in that country by conventional PCR (Dita *et al*., 2010; Li *et al*., 2015) and qPCR tests using the Clear Detection Kit™. The studies cited above, which have used various molecular identification methods, indicate that the *Foc*TR4 can be accurately identified based on resources available in most countries.

Regarding pathogenicity, except for *F. solani*, the *Fusarium* species included in the pathogenicity tests of the present study (*F. oxysporum, F. oxysporum* f. sp. *cubense*, and *F. sacchari*) caused mild to severe necroses in the rhizomes of inoculated plants 40 d after inoculation. Discolouration in rhizome cores confirmed the pathogenicity of *Fusarium* isolates identified as *Foc*TR4 and its association with wilt, while the other isolates were associated with root and collar rot. This study is the first to report *F. oxysporum* and *F. sacchari* causing root necrosis, collar and rhizome rots in Türkiye. Maryani *et al.* (2019b) examined *Fusarium* diversity in banana plants in small holder fields in Indonesia, and 90% of the *Fusarium* species isolated from pseudostems were in the *F. oxysporum* species complex, but members of the *F. incarnatum-equiseti*, *F. fujikuroi* and *F. sambucinum* species complexes were also recovered. In their pathogenicity tests, none of the Indonesian *Fusarium* species belonging to these species complexes (including *F. sacchari*) were found to be associated with Fusarium wilt in 'Cavendish' banana plants. The present study results agree with the pathogenicity results of Maryani *et al.* (2019b), but the present study *F. sacchari* isolates caused root collar and rhizome rots. Therefore, *F. sacchari* should not be overlooked in banana plantations. In addition, Cui *et al.* (2021) highlighted horizontal gene transfer between *Fusarium* species, indicating that the potential of *F. sacchari* to cause wilt in bananas should be carefully monitored.

Fusarium oxysporum isolates caused severe symptoms, although not as severe as those caused by *Foc*TR4 isolates. Rhizome necroses started from outside and progressed towards the plant cores, suggesting that *F. oxysporum* isolates also cause root and root collar rot, but not wilt. *Fusarium oxysporum* is an important species, with numerous *formae speciales* and physiological races, now associated with wilt, root/crown rot, damping-off, head blight, and seed/fruit rots in many plants (Edel-Hermann and Lecomte, 2019). The host specificity of *F. oxysporum*, and the fact that some isolates have endophytic or pathogenic characteristics, indicate that the fungus has a complex genetic diversity (Lombard *et al.,* 2019). Alteration of pathogenic characteristics by horizontal gene transfer between isolates has also been reported in *F. oxysporum* (Vlaardingerbroek *et al*., 2016). In pathogenicity tests by Wu *et al.,* (2019), using the 'Cavendish' (AAA) cultivar, only *Foc*TR4 isolates

induced rhizome pith necroses, whereas this symptom was not observed in plants inoculated with *Foc*1 isolates.

The present study has revealed that Fusarium wilt caused by *Foc*TR4 was present but had low prevalence (6.8%) in Turkish banana plantations. Since this race was detected in bananas in closed plastic protected greenhouses, legal and technical requirements have been fulfilled to eradicate the disease in these areas. In addition to wilt caused by *Foc*TR4, some *F. oxysporum* and *F. sacchari* isolates used in this study were found to cause root and collar rots, and, eventually, death of banana seedlings. This is the first report of *F. sacchari* associated with root and collar rot of banana in Türkiye. More research is required to enable rapid and accurate pathogen detection, and to restrain the re-emergence and spread of these diseases in this country.

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