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Tomato Response to *Fusarium* spp. Infection under Field Conditions: Study of Potential Genes Involved

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Abstract: Tomato is one of the most important horticultural crops in the world and is severely affected by Fusarium diseases. To successfully manage these diseases, new insights on the expression of plant–pathogen interaction genes involved in immunity responses to *Fusarium* spp. infection are required. The aim of this study was to assess the level of infection of *Fusarium* spp. in field tomato samples and to evaluate the differential expression of target genes involved in plant–pathogen interactions in groups presenting different infection levels. Our study was able to detect *Fusarium* spp. in 16 from a total of 20 samples, proving the effectiveness of the primer set designed in the ITS region for its detection, and allowed the identification of two main different species complexes: *Fusarium oxysporum* and *Fusarium incarnatum-equiseti*. Results demonstrated that the level of infection positively influenced the expression of the transcription factor *WRKY41* and the *CBEF* (calcium-binding EF hand family protein) genes, involved in plant innate resistance to pathogens. To the best of our knowledge, this is the first time that the expression of tomato defense-related gene expression is studied in response to Fusarium infection under natural field conditions. We highlight the importance of these studies for the identification of candidate genes to incorporate new sources of resistance in tomato and achieve sustainable plant disease management.

Keywords: *Solanum lycopersicon*; Fusarium diseases; fungal infection; disease resistance; gene expression

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1. Introduction

Tomato (*Solanum lycopersicon*) is considered one of the most important and widespread horticultural crops in the world [1,2]. In Portugal, tomato is one of the main vegetable crops for fresh consumption, and the main crop for horticulture industry, with an estimated production near 1.4 million tonnes and a harvested area of 15,040 ha in 2020 (https://www.fao.org/faostat/en/#data/QCL/visualize, accessed on 23 February 2022). The cultivation of tomatoes for industry, in Portugal, is mostly distributed throughout Ribatejo, Douro Valley, Sorraia Valley and some irrigated areas of Alentejo, while tomatoes for fresh consumption are mostly produced in Ribatejo, Algarve and Entre-Douro-e-Minho [3].

Tomato is affected by numerous diseases caused by many different agents including fungi, fungus-like organisms, bacteria, viruses and phytoplasmas, as well as physiological disorders, responsible for symptoms including fruit spots, rots, wilts, and leaf spots/blights [4,5]. Fungal diseases have high impact on tomato production and amongst them we highlight those caused by *Fusarium* spp. fungi as responsible for severe yield losses throughout the world. These are able to infect tomato plants by spore germination or

mycelium, resulting in higher plant transpiration and lower nutrient translocation, causing wilting, crown and root rot and, ultimately, death of the plant [6,7].

Fusarium oxysporum is a worldwide spread and phylogenetically diverse species, well known as a mycotoxin producer [8], and is considered as the most frequent species causing wilts, as well as crown and root rot, in different crops. Nevertheless, other Fusarium species have been constantly evolving and increasingly associated with many wilt diseases affecting different vegetables including bell pepper, chili pepper, cauliflower, sweet pepper, onion, potato, tomato and many others [9–11]. For instance, a recent study reports that *F. equiseti* is an important pathogen that is capable of reaching epidemic proportions that may seriously affect tomato cultivation in the future [6].

Considering *F. oxysporum* species, the two main formae speciales are *F. oxysporum* f. sp. *lycopersici* (FOL) and *F. oxysporum* f. sp. *radicis-lycopersici* (FORL). These formae speciales display genetic, epidemiological and symptomatologic differences; however, they are very difficult to discriminate by morphological and physiological features [12,13]. FOL is responsible for Fusarium wilt, and FORL causes Fusarium crown and root rot, which are among the most intensively studied plant diseases. Both formae speciales cause extensive production losses in tomato fields and greenhouses, being considered as limiting factors for tomato production, despite the current management techniques available [14].

Even though there are many management strategies that can prevent or reduce Fusarium diseases, most are harmful to the environment or not effective. In this way, the development and use of resistant plants is an alternative to these products. Resistant cultivars can be created by traditional breeding or by using genome editing approaches [15]. Recent technological developments, such as transcriptome analysis, has increased the knowledge on the molecular mechanisms involved in plant–pathogen interaction. In fact, the identification of plant key functional genes in susceptible responses and the understanding of the molecular basis of compatible interactions is possible using techniques that allow the study of differential gene expression [16]. Therefore, this knowledge is crucial to successfully manage these diseases, favoring the plants resistance [14,17,18].

Previous transcriptome studies on plant resistance mechanisms against *Fusarium* spp. infection already allowed the identification of important gene families involved. For instance, transcription factors (TFs) have been described to play a key role in plant immunity [19,20]. Among TFs, the WRKY family is reported to interact with pathogen-associated molecular patterns (PAMPs) or effectors to activate or repress, respectively, PAMP-triggered immunity (PTI), the first stage of defence, and effector triggered immunity (ETI), which is able to activate several defensive mechanisms such as the hypersensitive response [21,22]. Additionally, WRKY TFs are also responsive to salicylic acid (SA) and jasmonic acid (JA), which are phytohormones involved in systemic acquired resistance [21–23]. *WRKY40* and *WRKY41* are among the TFs previously described as being induced in tomato roots infected by FOL. *WRKY41* was only induced in a resistant cultivar, however, *WRKY40* was induced in both resistant and susceptible cultivars studied [24].

Receptor like-protein kinases (RLK) are pattern recognition receptors (PRRs) and were already referred to as involved in PTI, recognizing PAMPs on the cell wall [25]. This leads to the production of reactive oxygen species, activation of mitogen-activated protein kinase cascades, G-proteins, ubiquitin, calcium, hormones, TFs, and epigenetic modifications that regulate the expression of pathogenesis-related (*PR*) genes [16,17]. Among *PR* genes, *PR1* are described to play an important role in abiotic and biotic stress responses in plants, being particularly involved in fungal resistance and SA pathways [19,26]. *PR1* was described as a SA response gene that was overexpressed in roots and leaves of tomato inoculated with *F. oxysporum* [27]. *PR1b* was described for being induced in a resistant cultivar of tomato infected by FOL [24].

Genes associated with the synthesis and transport of calcium, such as *CBEF* (calcium binding protein EF hand family domain) and *CNGC* (cyclic nucleotide gated channels), also play an important role in PTI and ETI, since changes in intracellular Ca^{2+} concentration were shown to correlate with the subsequent defence-related physiological responses [19,28,29].

CBEF was suppressed for both resistant and susceptible cultivars of tomato infected by FOL, while *CNGC* was induced only in the resistant cultivar [24].

Regarding genes involved in the biosynthesis of JA, *OPR3* (12-oxophytodienoate reductase 3) has been studied for its role in mycorrhizal-induced resistance against *F. oxysporum* in tomato plants [27].

Considering the importance of tomato worldwide, and since there is a necessity of finding new sustainable strategies against diseases caused by *Fusarium* spp., responsible for huge production losses, the study of plant resistance mechanisms is crucial. Therefore, understanding transcriptional responses is of major importance for the comprehension of disease dynamics, with the modulation of gene transcription being an essential step for an efficient defence response in host cells [21]. The present study intends to test the hypothesis that different levels of infection of *Fusarium* spp. cause differential expression of defense-related genes in tomato plants growing under field conditions.

To the best of our knowledge, this is the first time that the expression of tomato defense-related genes expression is studied in response to Fusarium infection under natural field conditions, since most studies on the subject use samples under controlled conditions.

2. Materials and Methods

2.1. Study Site and Sampling

Sampling area is located in Salvaterra de Magos, in the Ribatejo region of Portugal (39°02′10.6″ N, 8°47′54.5″ W). This field was intensively cropped with tomato and known to be infested with Fusarium diseases for many years. Samples were collected in mid-June 2021, and weather conditions were characterized by high temperature as well as a high relative moisture, since this was an irrigated field. In this region, during May and June, maximum temperatures varied from 17 °C to 35 °C, and minimum temperatures varied from 7 °C to 16 °C (https://www.meteoblue.com/pt/tempo/historyclimate/weatherarchive/alverca-do-ribatejo_portugal_2271797, accessed on 23 March 2022).

The sampled tomato plants belonged to the H1534 variety, from Heinzseed (Stockton, CA, USA), which has mid-season maturity and can grow under humid or arid conditions. This variety is highly resistant to several diseases including Fusarium wilt caused by FOL races 1, 2 and 3 [30].

A total of 20 tomato plants were randomly collected from the experimental field, eight weeks after plantation. Samples were collected in the early morning, to minimize abiotic stress conditions, and were immediately transported to the laboratory where they were processed as promptly as possible. Plant crowns were detached and surface disinfected, according to Varanda et al. [31], and ground into powder, separately for each sample, using sterile mortars and pestles, aiding the process with liquid nitrogen. Ground plant materials were stored at −80 °C until further processing for both genomic DNA (gDNA) and RNA extraction.

2.2. Evaluation of Fusarium spp. Infection Level in Tomato Plants

2.2.1. gDNA Extraction

gDNA extraction was performed from approximately 500 mg of material powder for each sample, using the CTAB (hexadecyltrimethylammoniumbromide) method [32] with some modifications [31]. The quantification of gDNA and the evaluation of its purity were determined in a Quawell Q9000 micro spectrophotometer (Quawell Technology, Beijing, China). All DNA samples were diluted to a final concentration of 100 ng/µL.

2.2.2. Real-Time Quantitative PCR (qPCR) Conditions for *Fusarium* spp. Detection and Quantification

Fusarium spp. detection and quantification was carried out by qPCR using a set of primers designed in the ribosomal internal transcribed spacer (ITS) region (Fw: 5′-AAAACCCTCGTTACTGGTAATCGT-3′; Rv: 5′-CCGAGGTCAACATTCAGAAGTTG-3′, amplicon size 69 base pairs) [33].

In order to confirm the specificity of the primers to *Fusarium* spp., a bioinformatic analysis was performed. For this purpose, we used the correspondent partial sequence from the ITS region of several Fusarium species, as well as other fungal species that affect tomato in the Mediterranean Basin identified by Panno et al. [18]. Analysis of the ITS sequences was based on a ClustalW Multiple alignment using BioEdit software [34] The search for homologous sequences was done using Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/ (accessed on 25 February 2022)).

Next, qPCR was performed using 200 ng of gDNA per sample, 10 µL of NZY qPCR Green Master Mix (2×) (Nzytech, Lisbon, Portugal) and 400 nM of each primer, for a total volume of 20 µL, on a LineGene9600Pus system (BIOER, Hangzhou, China). Threshold cycle (Ct) values were acquired, for each sample, with the following cycling conditions: 20 s at 95 °C for an initial denaturation, followed by an amplification program of 40 cycles of 15 s denaturation at 95 °C and 20 s at 60 °C. Additionally, a final step was added to the program to test PCR specificity, a dissociation curve, featuring a single cycle at 95 °C for 15 s, 60 °C for 1 min and rump-up 0.2 °C/s to 95 °C for 15 °C. Three technical replicates were considered for each sample and *Fusarium* spp. isolates (including *F. oxyporum* f. sp. *radicis-lycopersici*, *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *cubense*, *F. incarnatum*, *F. equiseti*, *F. graminearum*, *F. verticillioides*, *F. subglutinans*, *F. proliferatum*, *F. sacchari* and *F. clavum*) from the collection of the Mycology Laboratory, Mediterranean Institute for Agriculture, Environment and Development (MED), University of Évora, Portugal, were used as positive controls. The identity of the amplicon of the samples was confirmed by Sanger sequencing and specificity of qPCR reactions was evaluated by melting curve analysis.

Considering the obtained Ct values, four groups of samples were set to study the target genes expression: group I (*Fusarium* spp. not detected), group II (Ct mean > 24), group III (22 < Ct mean < 24) or group IV (Ct mean < 22).

2.3. Target Genes Expression

2.3.1. RNA Extraction and Complementary DNA (cDNA) Synthesis

RNA was extracted following the RNeasy Plant Mini Kit (Qiagen) protocol. The quantification of RNA and the evaluation of its purity were determined in Quawell Q9000 micro spectrophotometer (Quawell Technology, Beijing, China). RNA integrity was evaluated by denature gel electrophorese. Total RNA (1000 ng) was reverse transcribed with the Maxima® First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA), in 20µL volume reactions, according to manufacturer's instructions. All cDNA samples were diluted to a final concentration of 5 ng/µL.

2.3.2. qPCR Conditions for Gene Expression Analysis

The genes considered for normalization were *TUB* (ß-tubulin), *ACT* (actin), *PGK* (phosphoglycerate kinase), *UBI* (ubiquitin-40S), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *PHD* (plant homeodomain finger family protein), *LSm7* (U6 snRNA-associated Sm-like protein LSm7) and *EXPRESSED* (expressed sequence uncharacterized protein). Primer sequences and amplicon sizes are shown in Table 1.

Target genes were chosen based on previous information on their involvement in resistance responses to *Fusarium* spp. [24,27]. Selected genes for gene expression study were: *WRKY 41*, *WRKY40*, *RLK*, *PR1b*, *CBEF*, *CNGC*, *OPR3* and *PR1* (Table 2).

Then, qPCR was performed using 10 ng of cDNA as template, 10 µL NZY qPCR Green Master Mix (2×) (Nzytech, Lisbon, Portugal) and 400 nM of each primer, for a total volume of 20 µL. The reactions were run on a LineGene9600Plus (BIOER, Hangzhou, China), with the following cycling conditions: 20 s at 95 °C for initial denaturation, an amplification program of 40 cycles at 95 °C for 15 s and 60 °C for 20 s. Additionally, a final step was added to the program to test PCR specificity, a dissociation curve, featuring a single cycle at 95 °C for 15 s, 60 °C for 1 min and rump-up 0.2 °C/s to 95 °C for 15 °C. Three technical

replicates were considered for each sample. Furthermore, primer efficiency was predicted by a five-point standard curve calculation from a five-fold dilution series (1:4–1:64) (run in triplicate) of pooled cDNA.

Table 1. Reference genes and primers used in qPCR.

Gene	Gene ID	Primer Sequence (5′ → 3′)	AS (bp)	R^2	E	Ref.
TUB	TC170178 [a]	F: CCTGGTGGTGACCTTGCTAAG R: CTCACCGACATACCAATGCAC	143	0.995	103.9	[35]
ACT	U60480 [b]	F: GGAATCCACGAGACTACATAC R: GGGAAGCCAAGATAGAGC	228	0.990	94.4	[35]
PGK	TC181003 [a]	F: TCTACAAGGCCCAAGGTTATG R: GCAGCAAACTTGTCCGCAATC	148	0.982	61.8	[35]
UBI	TC193502 [a]	F: GGACGGACGTACTCTAGCTGAT R: AGCTTTCGACCTCAAGGGTA	134	0.995	90.7	[36]
GAPDH	TC198136 [a]	F: CTGCTCTCTCAGTAGCCAACAC R: CTTCCTCCAATAGCAGAGGTTT	156	0.998	92.3	[36]
PHD	Solyc06g051420.2.1 [c]	F: GGGATGGGATGGAGCGTAGAGA R: CATCACTCTCCTCTTGCAGCCT	279	0.944	71.8	[36]
LmS7	Solyc09g009640.2.1 [c]	F: GGTGGAAGACAAGTGGTTGGAACAC R: CGTCTGGCTGAACAAAAGGATTGG	220	0.997	99.9	[36]
EXPRESSED	Solyc07g025390.2.1 [c]	F: GCTAAGAACGCTGGACCTAATG R: TGGGTGTGCCTTTCTGAATG	183	0.999	95.0	[37]

[a] accession number at TIGR; [b] accession number at NCBI; [c] accession number at Sol Genomics Network. AS: amplicons size; R^2: coefficients of determination; E: primer efficiency.

Table 2. Target genes and primers used in qPCR.

Gene	Gene ID	Primer Sequence (5′ → 3′)	AS (bp)	R^2	E	Ref.
WRKY41	Solyc01g095630 [a]	F: TCCTCATTTGGTGGAGAAGG R: TAGCTTAGGATCAATTAGGC	171	0.997	102.0	[24]
WRKY40	Solyc06g068460 [a]	F: GAGTTGGCTAGATTGAGACTG R: TTGATGCCACAAAAGAGTTG	144	0.999	97.0	[24]
RLK	Solyc03g059080 [a]	F: GCAGTGTGTAGATCCTAAGC R: CAGTGCCTTGACGACAATTG	210	0.995	103.2	[24]
PR1b	Solyc00g174340 [a]	F: ATACTCAAGTAGTCTGGCGC R: GTAAGGACGTTGTCCGATCC	106	0.972	98.2	[24]
CBEF	Solyc10g006660 [a]	F: ATTAAGTCCTGAGTTGATGG R: GATAACAGTGCATCAGAAGGG	107	0.951	110.8	[24]
CNGC	Solyc05g050350 [a]	F: CACAAATGCATCAAGTCTTGG R: CTAAAATCTGGTTCAGCTGG	141	0.991	103.3	[24]
OPR3	NM_001246944 [b]	F: CCTCTTTCAAACAACAATGGCG R: TGAACTTGCCCATCTTGTAAGG	115	0.996	101.4	[27]
PR1	EU589238 [b]	F: GCAGCTCGTAGACAAGTTGGAGTCG R: TGTTGCATCCTGCAGTCCCC	107	0.995	100.8	[27]

[a] accession number at Sol Genomics Network; [b] accession number at NCBI. AS: amplicons size; R^2: coefficients of determination; E: primer efficiency.

Evaluation of expression stability of the reference genes and selection of the most appropriate combination of genes to be used for data normalization was done using the statistical application geNorm [38]. To study target gene expression, Ct values were regressed on the log of the previously constructed cDNA curve. Subsequently, the value of normalized arbitrary units of the target genes, for each sample, was calculated using normalization factors obtained for the reference genes.

2.3.3. Statistical Analysis

The relative expression of target genes (*WRKY41*, *WRKY40*, *RLK*, *PR1b*, *CBEF*, *CNGC*, *OPR3* and *PR1*) between the different levels of infection by *Fusarium* spp. were subject to a uni and multivariate permutational analysis of variance (PERMANOVA) with PRIMER v6 software [39] add-on package [40] to find significant differences ($p < 0.05$). A one-way PERMANOVA was performed to test the hypothesis that significant differences occurred within the "levels of infection", and the one-factor design: "levels of infection", group I, group II, group II and group IV (one level, fixed) were used. A Bray–Curtis similarity matrix [41] was always used on PERMANOVA analysis, and Monte Carlo permutation p was carried out in case of the number of permutations was lower than 150. Whenever significant interaction effects were detected, these were examined performing a posteriori pairwise comparisons, under a reduced model using 9999 permutations [42,43].

3. Results

3.1. Evaluation of Fusarium Infection Level in Tomato Plants

Tomato field samples were tested in order to detect the presence of *Fusarium* spp. following qPCR approach, using a pair of primers that specifically target *Fusarium* spp. The specificity of the primers to *Fusarium* spp. can be verified in the alignment of Figure 1, in which we compared a partial sequence of the ITS region of nuclear rDNA of several Fusarium species, as well as other fungal species that affect tomato in the Mediterranean Basin identified by Panno et al. [18]. The analyzed region allows the easy discrimination of *Fusarium* spp. from other fungi. Isolates from all *Fusarium* spp. analyzed were identified using these primers, despite the lack of full homology in these regions (differences in few nucleotides).

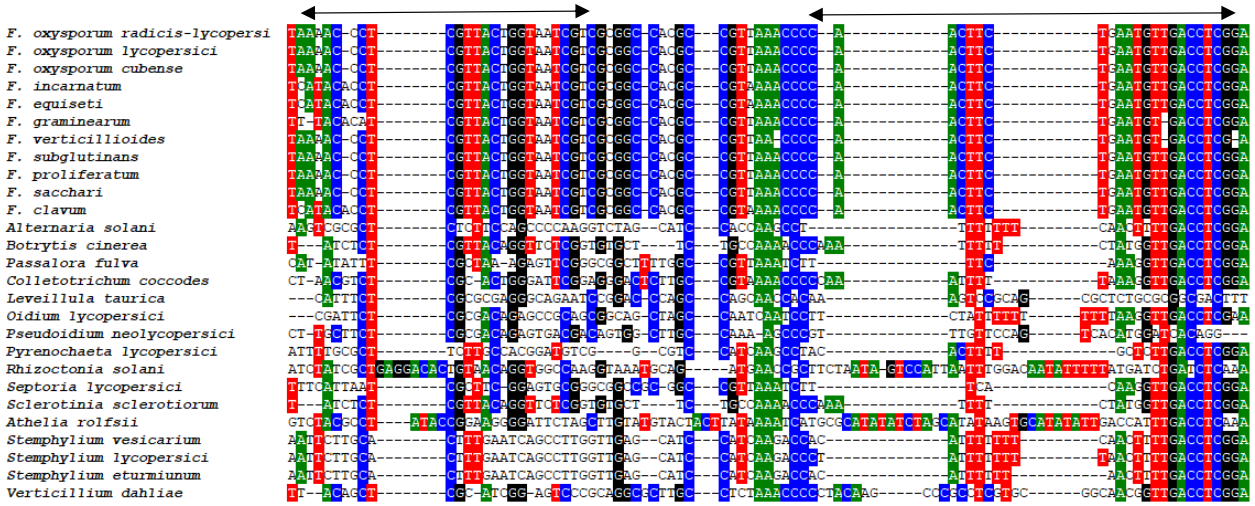

Figure 1. Multiple alignment of a partial sequence of the ribosomal internal transcribed spacer (ITS) region of nuclear rDNA of Fusarium species and other fungal species that affect tomato in the Mediterranean Basin. Arrows indicate the location of the primers used on qPCR. Accession numbers to published sequences in the GenBank are as follows: *F. oxysporum* f. sp. *radicis-lycopersici* (MH865886.1); *F. oxysporum* f. sp. *lycopersici* (MH458918.1); *F. oxysporum* f. sp. *cubense* (MH681692.1); *F. incarnatum* (MW489422.1); *F. equiseti* (MW785181.1); *F. graminearum* (MH054937.1); *F. verticillioides* (KT357570.1); *F. subglutinans* (OM185557.1); *F. proliferatum* (MW704332.1); *F. sacchari* (OL347721.1); *F. clavum* (MZ890488.1); *Setophoma terrestris* (OL960208.1); *Alternaria solani* (OK427286.1); *Botrytis cinerea* (MW301135.1); *Passalora fulva* (KF876173.3); *Colletotrichum coccodes* (OL831117.1); *Leveillula taurica* (OK036585.1); *Oidium lycopersici* (AF229021.1); *Pseudoidium neolycopersici* (AB163916.1); *Pseudopyrenochaeta lycopersici* (MK052946.1); *Pyrenochaeta lycopersici* (AM944362.1); *Rhizoctonia solani* (MW498395.1); *Septoria lycopersici* (KF251463.1); *Sclerotinia sclerotiorum* (MF563992.1); *Athelia rolfsii* (MW349665.1); *Stemphylium vesicarium* (MZ099818.1); *Stemphylium lycopersici* (MZ093130.1); *Stemphylium eturmiunum* (MZ093121.1); *Verticillium dahliae* (GU060637.1).

From the total of 20 sampled tomato plants, 16 were infected by *Fusarium* spp. Ct mean values and standard error (±SE) are shown in Table 3 for each tomato plant.

Table 3. Mean threshold cycle values (Ct) and standard error (±SE) of tomato sampled plants infected with *Fusarium* spp. Transcript levels were determined by qPCR.

Sample	Ct Mean	±SE
1	ND	-
2	21.34	±0.095
3	26.38	±0.022
4	23.16	±0.015
5	23.77	±0.150
6	24.62	±0.060
7	ND	-
8	25.01	±0.425
9	23.36	±0.000
10	21.24	±0.005
11	22.37	±0.200
12	21.47	±0.055
13	24.11	±0.085
14	21.98	±0.145
15	ND	-
16	23.38	±0.170
17	ND	-
18	22.28	±0.010
19	17.8	±0.090
20	24.04	±0.031

ND: not detected.

Evaluation of melting curve analysis confirmed specificity of qPCR reactions and Sanger sequencing allowed validation of the identity of amplicons obtained. Sequencing of the qPCR products allowed the identification of two main different species complexes in the tomato samples: *Fusarium oxysporum* (FOSC) and *Fusarium incarnatum-equiseti* (FIESC).

In order to differentiate tomato plants according to levels of infection, four different groups were established (see Materials and Methods, Section 2.2.2). We chose five samples from each group of infection level as replicates and four samples from group I, since these were the only samples in which *Fusarium* spp. was not detected.

3.2. Target Gene Expression

From a total of eight tomato reference genes considered for normalization (Table 1), *TUB* and *UBI* were the most stable genes tested and were, therefore, selected to normalize target gene expression. The target genes were chosen based on previous information on their involvement in plant resistance responses to *Fusarium* spp., as follows: *WRKY40*, *WRKY41*, *RLK*, *PR1b*, *PR1*, *CBEF*, *CNGC* and *OPR3* (Table 2). PCR efficiency values and correlation coefficients (R^2) for reference genes and target genes tested are presented on Tables 1 and 2, and amplification plots are presented in Figure S1.

Statistical analysis on the different levels of infection for each target gene allowed comparisons of gene expression values (normalized arbitrary units). This analysis demonstrated significant differences in gene expression between different levels of infection for *WRKY41* and *CBEF* genes (Figure 2). For the remaining target genes, no significant differences were detected in their expression when the levels of infection were compared (Figure 2).

Considering the *WRKY41* gene, the mean ± SE of the the arbitrary unit values was 0.65 ± 0.12 for group I of infection level, 0.81 ± 0.07 for group II, 2.24 ± 0.83 for group III and 1.82 ± 0.42 for group IV. PERMANOVA analysis, for the arbitrary unit values of the *WRKY41* gene, revealed significant differences (p = 0.0158) in the factor 'levels of infection'. Individual pairwise comparisons detected significantly higher arbitrary unit values of this gene for group III than for groups I and II (pairwise tests, $p_{\text{I vs. III}} = 0.0361$; $p_{\text{II vs. III}} = 0.0457$).

Significantly higher arbitrary unit values were also found for group IV than for groups I and II (pairwise tests, $p_{I\ vs.\ IV} = 0.0249$; $p_{II\ vs.\ IV} = 0.0316$). In addition, individual pairwise comparisons revealed no significant differences between groups I and II (pairwise tests, $p_{I\ vs.\ II} = 0.2578$) and between groups III and IV (pairwise tests, $p_{III\ vs.\ IV} = 0.9061$) (Figure 2).

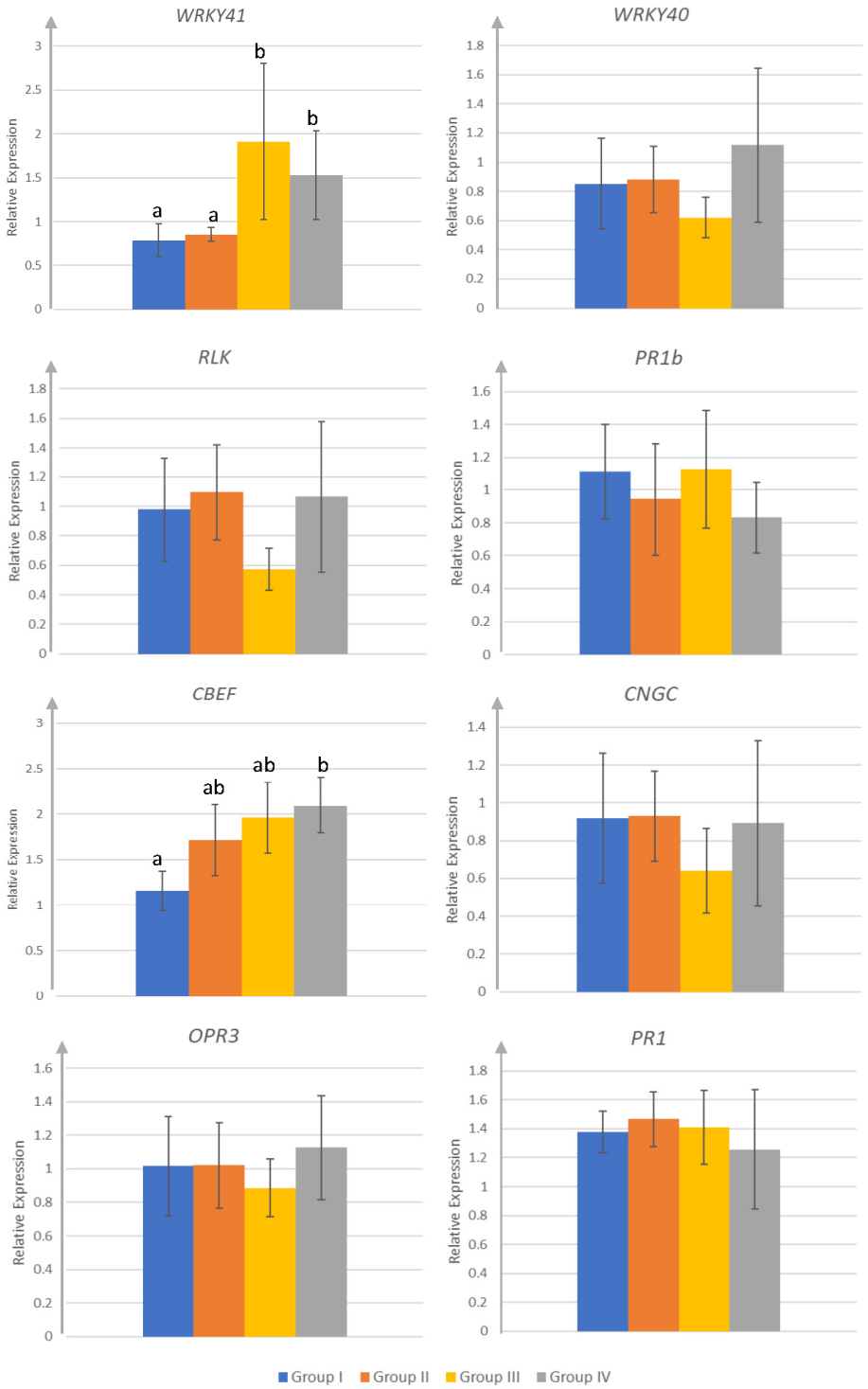

Figure 2. Transcript accumulation of *WRKY41*, *WRKY40*, *RLK*, *PR1b*, *CBEF*, *CNGC*, *OPR3* and *PR1*, upon different levels of *Fusarium* spp. infection (group I, group II, group II, group IV) growing under field conditions. Transcript levels were determined by qPCR. Error bars indicate the standard error of the mean. Significant differences are identified with different lowercase letters.

For the *CBEF* gene, the mean ± SE of the the arbitrary unit values was 1.16 ± 0.21 group I of infection level, 1.71 ± 0.39 for group II, 1.96 ± 0.39 for group III and 2.10 ± 0.24 for group IV. PERMANOVA analysis, for the arbitrary unit values of the *CBEF* gene, did not reveal significant differences ($p = 0.2148$) in the factor 'levels of infection'. Individual pairwise comparisons only detected significantly higher arbitrary unit values of this gene in group IV, compared to group I (pairwise tests, $p_{I\ vs.\ IV} = 0.0168$). In addition, individual pairwise comparisons did not detect significant differences between the other levels of infection (pairwise tests, $p_{I\ vs.\ II} = 0.4192$; $p_{I\ vs.\ III} = 0.0893$; $p_{II\ vs.\ III} = 0.5854$; $p_{II\ vs.\ IV} = 0.3312$ and $p_{III\ vs.\ IV} = 0.6074$) (Figure 2).

4. Discussion

Identification and quantification of *Fusarium* spp. in tomato plants was essential in order to evaluate the infection level of this fungi in field samples. Since tomato crop is affected by numerous diseases caused by many different agents including fungi, fungus-like organisms, bacteria, viruses, phytoplasmas, as well as physiological disorders [4], the identification of a specific genus, particularly in field samples, proves to be a great challenge, requiring the development of rapid and accurate methods for its detection and diagnosis [44]. In this study, we were able to successfully detect *Fusarium* spp. and discriminate this genus from other fungi that affect tomato, using a specific set of primers and following a qPCR approach [33]. Evaluation of melting curve analysis confirmed specificity of qPCR reactions, and Sanger sequencing allowed validation of the identity of the amplicons obtained, confirming the identification of *Fusarium* spp. in tomato plants. Furthermore, ITS regions have been previously and effectively used to generate specific primers able to differentiate closely related fungal species [33,45–48]. In view of the described above, we proved the effectiveness of the primer set designed in the ITS region for the detection of *Fusarium* spp. in tomato field samples.

Considering that the studied tomato field has been known to be intensively cultivated with tomato and infested with Fusarium diseases for many years, our results demonstrated that, as expected, most samples were infected with *Fusarium* spp. It is difficult to manage Fusarium diseases in fields affected for many years, since these fungi are very well adapted and can survive under extreme conditions, disseminating by conidia, in tomato seeds and seedlings, soil and other media, and even irrigation water [14].

Although the cultivar used in this experiment is highly resistant to several diseases including Fusarium wilt caused by FOL races 1, 2 and 3 [30] (see the Materials and Methods section), it was expected that other Fusarium species might exist in the experimental field. In fact, sequencing of the qPCR products allowed the identification of two main different species complexes: FOSC and FIESC. Despite *F. oxysporum*, especially FOL and FORL, being considered as the most frequent disease agents in tomato [12–14], additional Fusarium species have been reported as pathogenic to some vegetables including tomato [9–11]. For instance, our results support the previously described information about *F. equiseti* as an important pathogen of tomato crop [6].

To study target gene expression, reference genes are needed as endogenous control. These genes must have high expression stability under the experimental conditions, allowing accurate relative quantification of target gene expression [35]. Even though relative quantification by qPCR widely relies on common internal control genes, the expression stability of some of these genes may vary in response to environmental conditions, which is even more likely to occur in field samples, exposed to a diversity of environmental conditions and plant–pathogen interactions [36,49–52]. Therefore, we reinforce the idea that the validation of reference genes for the conditions under study is essential.

The target genes selected were based on previous transcriptome studies on tomato resistance responses to *Fusarium* spp. infection that had already identified some important gene families involved [24,27]. These and other studies on the subject have revealed quite variable transcriptional responses, depending on the plant and pathogen species involved, as well as on plant cultivars and their specific characteristics [16,19,20,24,26], highlighting

the importance of these studies for identification of candidate genes in view of plant breeding [16,53].

Target gene expression confirmed a significantly different expression of *WRKY41* and *CBEF* genes, indicating that the level of infection has influence on the expression of the TF *WRKY41* and on the calcium-binding EF hand family protein, suggesting their involvement in tomato immunity responses to *Fusarium* spp. (Figure 2). However, infection level did not influence the expression of the remaining target genes studied, contrasting information formerly attained for *WRKY40*, *RLK*, *PR1b*, *CNGC* and *CBEF* [24], but supporting the previously described for *OPR3* and *PR1* [27]. Contrasting results might be explained by the high variability found in the expression of a specific gene within the same group of infection level, noted on the ±SE values (Figure 2). Nevertheless, this variability was not totally unexpected, since the work presented here was developed with samples under field conditions, exposed to diverse environmental constrains. Under these conditions, *Fusarium* spp. inoculum is probably not equally distributed in the field, and it may not be the same species for all extension, meaning that different species of Fusarium might be found in different samples.

Furthermore, most of the previous studies compare resistant vs. susceptible tomato cultivars and were performed under controlled conditions, with inoculation of specific pathogens [16,19,20,24,26,27]. On the other hand, the present study investigates, under field conditions, and using a single cultivar, if different levels of infection influence differential expression of target genes involved in tomato resistance responses to *Fusarium* spp. Therefore, we present an approach to get new insights on plant–pathogen interactions in real field conditions, based on levels of infection.

As stated above for the *WRKY41* gene, our results showed differential expression for different levels of infection by *Fusarium* spp. Samples from groups III and IV, with higher infection levels, showed significantly higher expression of *WRKY41* gene than samples from groups I and II, with no infection and the lowest level of infection, respectively. Our data proves that *WRKY41* gene is induced by higher levels of infection by *Fusarium* spp., being in agreement with the results present by Zhao et al. [24], which identified this gene as induced in the resistant cultivar of tomato infected by FOL. Additionally, *WRKY41* has been identified for playing a positive role in defense activation and host resistance to other pathogens, such as *Oidium neolycopersici* in wild tomato, being possibly induced by SA and/or ethylene [54].

TFs have been reported as having important roles on the regulation of gene expression in the response of plants to abiotic and biotic stresses, with the *WRKY* TF gene family being one of the most important involved in plant immunity responses [19–21]. *WRKY* genes are responsive to pathogens, elicitors and SA and JA phytohormones, being able to positively or negatively regulate several aspects of the plant innate immune system, which consists of PTI and/or ETI [21,22]. In tomato, a total of 83 WRKYs have been identified [55] and, in addition to *WRKY41*, other genes have been recognized for tomato-pathogen interactions: *WRKY45* for enhancing tomato susceptibility to the root-knot nematode *Meloidogyne javanica* [56]; *WRKY39* for enhancing resistance to *Pseudomonas syringae* [57]; *WRKY33* for enhancing resistance to hemibiotrophic oomycete *Phytophthora infestans* [54]; *WRKY9*, *WRKY6*, *WRKY36* and *WRKY37* for being upregulated in plants inoculated with FOL [19,58].

Results obtained for the *CBEF* gene showed significantly higher expression of the gene for the higher level of infection (group IV) compared to its expression on samples with no infection (group I). According to our results, the *CBEF* gene is induced upon high level of infection by *Fusarium* spp., which does not agree with previous results described by Zhao et al. [24], that showed the *CBEF* gene as being supressed in both resistant and susceptible tomato cultivars infected by FOL. Differences might be related to the cultivars used, which are not the same, the Fusarium species affecting tomato plants and the defence mechanisms being activated, once more revealing the diversity of transcriptional responses that might occur [16,19,20,24,26]. Nevertheless, genes associated with the synthesis and

transport of calcium, such as the *CBEF* gene, are known to play an important role in PTI and ETI, since changes in intracellular Ca^{2+} concentration were shown to correlate with the subsequent defence-related physiological responses [19,28,29].

5. Conclusions

The present study highlights the importance of new knowledge on the molecular mechanisms involved in tomato–pathogen interactions and the novelty of studying gene expression in plant samples growing under field conditions. Our results reveal that the level of infection caused by *Fusarium* spp. positively influences the expression of *WRKY41* and *CBEF* genes. The identification of candidate genes involved in plant–pathogen interactions will facilitate genetic engineering efforts to incorporate new sources of resistance against pathogens in tomato. These genes might be promising candidates for strategies involving gene knockout or overexpression, offering extended and sustainable possibilities to successfully manage Fusarium diseases in tomato, relying on the plant's innate immune mechanisms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8050433/s1, Figure S1: Real time quantitative PCR amplification plots to access the expression of the tomato genes *WRKY 41*, *WRKY 40*, *RLK*, *PR 1 b*, *CBEF*, *CNGC*, *OPR 3* and *PR 1* upon *Fusarium* spp. infection.

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