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Quantitative Evaluation of Chickpea Fusarium Wilt

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ABSTRACT

***Fusarium Oxysporum* f. sp. *Ciceris* (FOC) is the causal agent of Fusarium wilt, a destructive and widespread disease of chickpea. Rapid and accurate identification and detection of plant pathogens are essential for timely Disease Management (DM) strategies with appropriate measures. This study aimed to quantitatively determine FOC by using Quantitative Real-Time Polymerase Chain Reaction (qPCR) technique with specific primer pairs [*Histone* (H3) and *Ribosomal* (J5)] in seed, root, and root collar, and to discriminate it from other pathogenic fungi [*Fusarium Oxysporum* formae speciales (FO f. sp.) and *Ascocyhta rabiei*]. Total RNAs were isolated, converted to cDNAs (limit of 5 ng/rxn.-0.05 pg/rxn.) and used as template for qPCR studies. The FOC was detected in plant samples starting from the first day after inoculation. The FOC was detected in root, root collar and seed samples and was differentiated by qPCR assay from other pathogenic fungi. Melting curves, in which no primer dimers and non-specific complementation were observed, presented a single peak. Quantification was successfully performed using specific H3 and J5 primer pairs (P< 0.05), and the FOC was distinguished from other pathogenic fungi with J5 primer (P< 0.05). The results of these studies may support the development of new biochemical and molecular methods that allow direct, faster and more accurate determination of pathogens. Thus, it will also enable us to reduce the losses caused by diseases and the costs of DM.**

Keywords: *Fusarium oxysporum* f. sp. *ciceris*, *Fusarium oxysporum* formae speciales, qPCR, RT-qPCR, Wilting and Yellowing.

INTRODUCTION

The south-eastern region of Turkey is a natural habitat for ancestral *Cicer* spp.. It assumes that chickpea (*Cicer arietinum* L.) was first domesticated in Fertile Crescent (Lev-Yadun *et al*., 2000; Abbo *et al*., 2003), and it has an important place for feed and food legume cultivation (Kafadar *et al*., 2019; Mart, 2022) that is preferred within the scope of sustainable agricultural and food security (Giri *et al*., 2023). However, phytopathogenic fungi, which cause various disease in crop plants, are responsible for serious yield losses since they adversely affect the hosts. The importance of crop production technologies is increasing in order to reduce the losses caused by diseases (Sharma *et al*., 2020).

Fusarium Oxysporum (FO) is an anamorphic fungal species called as the species complex, consisting of many strains with similar morphology, including pathogenic and non-pathogenic forms (Kumar, 2021). It is one of the major plant pathogens with a wide host range, grouped into formae speciales (ff. sp.) depending on their host range (Ates *et al*., 2019).

Fusarium wilt caused by the agent *Fusarium Oxysporum* Schlecht. emend. Snyd. & Hans. f. sp. *Ciceris* Matuo & K. Sato (FOC), is one of the most common and

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destructive disease of chickpea (Jendoubi *et al*., 2017), resulting in serious yield losses in the world as well as Turkey (Kocalar *et al*., 2020). The disease is seed and soil-borne (Jendoubi *et al*., 2017), has two different disease forms, namely, wilting (early plant death) and yellowing (late plant death) (Kocalar *et al*., 2020; Singh and Vyas, 2021). The yellowing form gradually occurs on the leaves and a change in vascular colour starting from the lower leaves, whereas the wilting form results in severe and rapid chlorosis, flaccidity in the leaves and shoots, followed by vascular discoloration and desiccation (Castro *et al*., 2010). Pathogen invasion begins at the roots and progresses to the xylem tissues preventing water and nutrient transport, ultimately leading to plant death.

Identification of FO strains is not easy due to the high degree of morphological and pathogenic variability among the same f. sp. Thus, it needs a quite laborious and time-consuming process that needs specialist to be involved, and detailed microscopic observation (Tekeoğlu *et al*., 2017). Traditionally, pathogenicity tests have been used together with current molecular techniques to distinguish pathogenic strains from non-pathogenic FO isolates, but they are not suitable for pathogens with much pathogenic diversity (Kumar *et al*., 2012). In addition, FO strains can gain and lose pathogenicity via transmission of chromosomes related to pathogenicity (Michielse and Rep, 2009).

There is wealth of information available on the infection process, virulence and crop protection of FO species complex-host interaction (Demirel *et al*., 2019; Başbağcı and Dolar, 2020). However, information on the precise quantification of fungal biomass in host tissues and its relation to disease severity is scarce.

Recently, new approaches are being developed and applied for morphological identifications of phytopathogenic fungi. New technological developments and applications also provide benefits for Disease Management (DM) (Singh *et al*., 2023), Sustainable Agriculture Practices (SAP) (Demirel *et al*., 2022; Scortichini, 2022) and Biological Control (BC) (Sahgal, 2022). Although the existing PCR based methods are applied for the determination of FO, the results do not provide quantification (Priyadarshini *et al*., 2021). Currently, the most reliable technique for *in*-vivo/*in*-vitro determination and quantification of various pathogens in plants is Quantitative Real-Time Polymerase Chain Reaction (qPCR) (Haegi *et al*., 2013). Commonly, total RNA isolation from infected plants was used to determine the expressions levels of particular gene of pathogen in the host during the infection process (Priyadarshini *et al*., 2023). However, information on the amount of pathogen determined by qPCR applications is still insufficient.

In this context, the aim of this research included: 1) rapid and reliable quantification of FOC in the infected chickpea tissues using primer pairs specific to the *Histone* (H3), and *ribosomal* (J5) gene regions, 2) to differentiate FOC (fungal agent) from other fungal pathogens [*Fusarium Oxysporum* formae speciales (FO ff. sp.), and *Ascocyhta rabiei*], and 3) to determine the specificity of designed primer pairs.

MATERIALS AND METHODS

Fungal and Plant Tissues, Inoculation, Scoring, and Storing

Within the study, Chickpea cultivar ILC482, which is susceptible to FOC, was inoculated with 4 different isolates chosen according to their virulence levels and disease forms (Kocalar *et al*., 2020). Disease scoring and plant sampling were carried out every three days after the 1st, 2nd, and 3rd day until the 31st day. Inoculation, scoring, and storing applications in the study are as stated in Demirel *et al*. (2019).

Plant tissues were cut with sterile scissors from randomly infected plants, wrapped in aluminium foil, and frozen in liquid nitrogen. All collected plant materials were

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kept at −80°C until Total RNA isolation (storing applications).

In addition to quantification of disease agent in inoculated plants, chickpea plants exhibiting disease symptoms were collected during survey studies, and direct quantification of fungi from seed, root and root collar plants (cultivars Azkan and Sezenbey) was performed. To test the specificity of the designed primer pairs, pure cultures of members of "*Fusarium Oxysporum* Species Complex (FOSC)" (Williams *et al*., 2016) other than FOC [*F. oxysporum* f. sp. *radicis-lycopersici*, *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *capsici* and *F. oxysporum* f. sp. *melongenae* (Altinok *et al*., 2018; Altınok *et al*., 2019; Ates *et al*., 2019) and *Ascochyta rabiei* were used (Polatbilek *et al*., 2017; Akveç *et al*., 2018).

RNA Isolation, cDNA Synthesis, and Quality Determination of Products

Total RNAs were isolated by using the PureLink RNA Mini Kit (12183018A, Invitrogen, USA) after homogenization of infected plant tissues with liquid nitrogen. The RNA concentration measurement was attained by a NanoDrop Spectrophotometer (MaestroGEN, Taiwan) and stored until next step at −20°C.

For Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR), complementary DNA (cDNA) synthesised from 2 µg total RNA using cDNA synthesis Kit (4368814, Applied Biosystems, USA).

Total RNA and cDNA of all samples were subjected to agarose gel electrophoresis stained with EtBr and visualized using a gel visualisation apparatus with a UV light source. The gel images were analyzed by ImageJ Analyse Program (Version 1.8.0 – National Institutes of Health, USA) and histograms were drawn.

qPCR Analysis

qPCR reaction (rxn.) of 20 µL volume consisted of 5 ng cDNA, SYBR Green PCR Master Mix (4367659, Applied Biosystems, USA), 0.5 µM of forward and reverse primers. The qPCR reaction was programmed to run for 10 min. at 95°C, for 40 cycles of 15 seconds at 95°C, and for 1 minute. at 60°C. Then, for the melting curve to be performed, 15 seconds at 95°C, 1 minute at 60°C, and 15 seconds at 95°C. Positive groups consisted of cDNAs converted from Total RNAs isolated from mycelial culture grown in PDB media and 6 different 10-fold dilutions (1:1, 1:10, $1:10^2$, $1:10^3$, $1:10^4$ and $1:10^5$).

qPCR assays were performed with 3 replicates in Applied Biosystem StepOnePlus in 96-well PCR plate with optical films. The primers used throughout the study are listed in Table 1. NCBI was used to arrive at the mRNA reference sequence accession number, FOC-specific primers are automatically designed by the IDT tool by selecting the best sequence based on GC content, Tm degrees, and base sequence length (quite short, like 15-30 nucleotides). Melting curve analysis was applied after qPCR reaction to test specificity of primers.

Table 1. Specific primers used in qPCR analysis.

Primer Name	Sequence (5'-3')	Accession Number
1H3_F	5'-TCTCCGCTTCCAGTCTTCT-3'	NC_030995
1H3_R	5'-AGCTGGATGTCCTTGGATTG-3'	
2J5_F	5'-GAGGCCGAGAAGAAGGTAAG-3'	JK747159
2J5_R	5'-GCCCAACTTGCGGTTAAAG-3'	

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Statistical Analysis

All the data were tested for normality and analyzed using the SPSS 22 statistical analysis program (IBM, 2013): One-Way ANOVA, Reliability test, Pearson correlation ($P< 0.01$ and $P< 0.05$), One sample t-test analyses were applied on data ($P< 0.01$ and $P< 0.05$) (Rieu and Powers, 2009; Zhu *et al*., 2016).

RESULT

Specificity and Reproducibility of qPCR Reactions

The total RNA and cDNA of all samples visualized by the agarose gel electrophoresis and analysed using ImageJ Analyse Program are given in Figures 1 and 2. The results of melting curve analysis applied after qPCR reactions of cDNA obtained from growth chamber studies are presented in Figure 3 and Table 2. A single sharp peak with an average melting temperature of 77.53°C for J5 primer and 83.42°C for H3 primer were observed after the melting curve indicator for high specificity of primers. However, more than one melting curve peaks were defined for fungal pathogens other than FOC, indicating high specificity of the qPCR reaction and primers for FOC (Figure 4 and Table 3). In addition, no peaks were observed in absence of cDNA of fungal agent. These results confirmed the high specificity of H3 and J5 primer pairs.

Quantitative Measurement

Primer pairs specific to histone (H3) and ribosomal (J5) genes of FO were used for

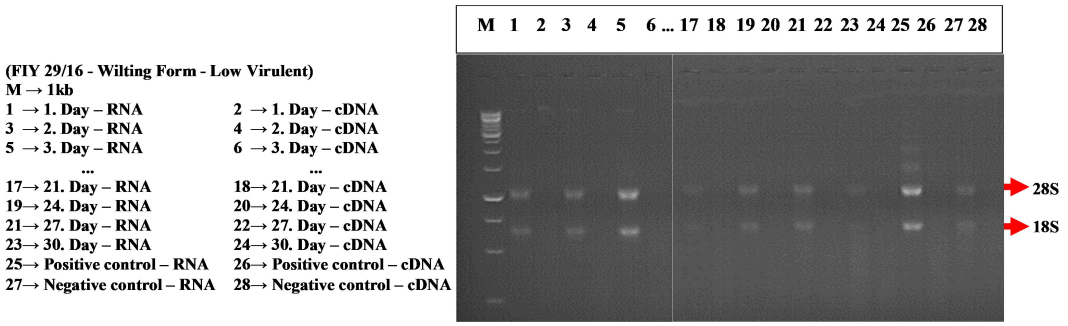

Figure 1. Agarose gel image of RNA isolated and cDNAs translated from the infected chickpea.

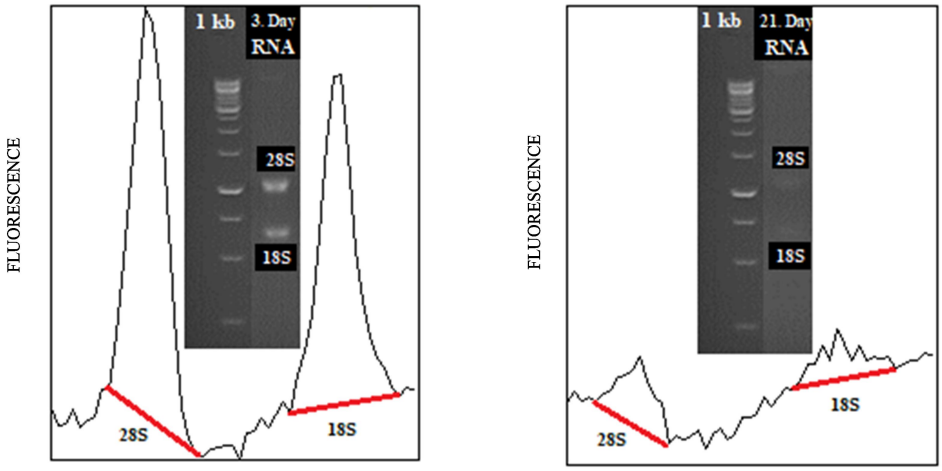

Figure 2. RNA quality assessed with ImageJ Profile Plot Program.

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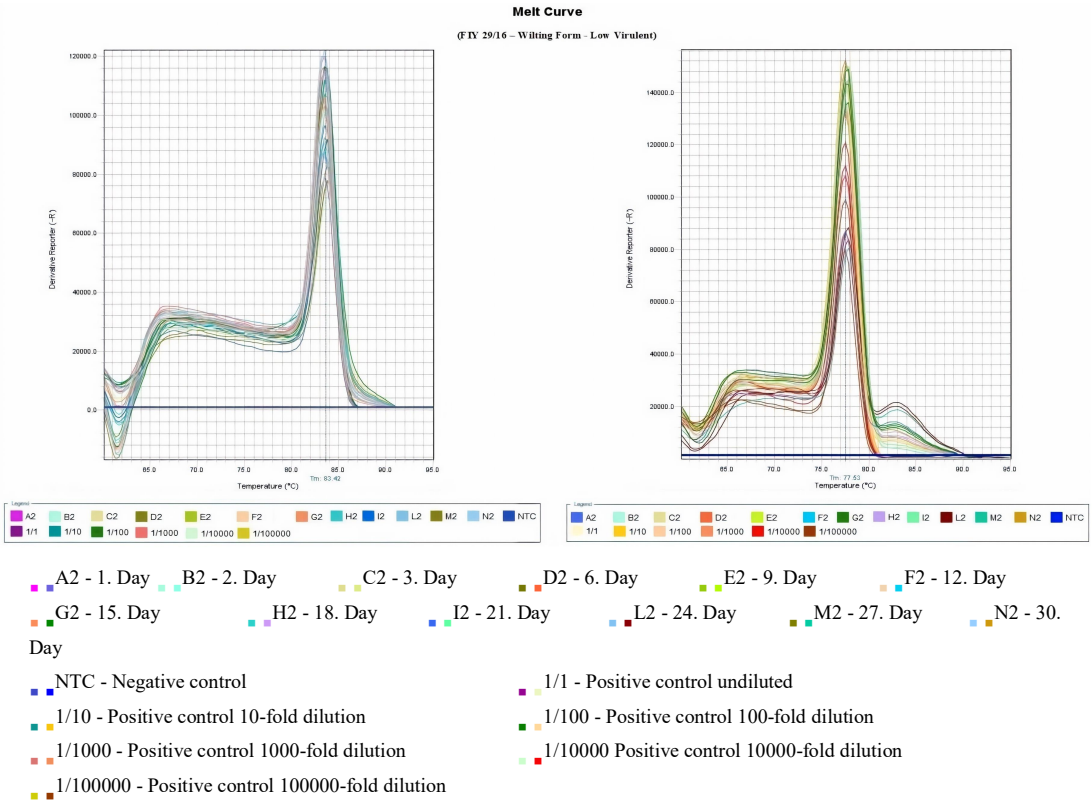

Figure 3. Melt Curve of infected plants tissues with wilting low virulence isolate in H3 (a) and J5 (b) primers.

Table 2. Melting temperatures for quantification of *Fusarium oxysporum* f. sp. *ciceris* by qPCR in low virulent wilting form samples.

Isolates	H3 (°C)	J5 (°C)	Isolates	H3 (°C)	J5 (°C)	Dilutions	H3 (°C)	J5 (°C)
A2	83.4237	77.5291	G2	83.4167	77.5205	1/1	83.4238	77.5508
A2	83.4237	77.5252	G2	83.4235	77.5345	1/1	83.4238	77.5251
A2	83.4237	77.5291	G2	83.4235	77.5454	1/1	83.4238	77.5251
B2	83.4203	77.5252	H2	83.4239	77.5280	1/10	83.4238	77.5333
B2	83.4203	77.5279	H2	83.4209	77.5280	1/10	83.4138	77.5306
B2	83.4203	77.5310	H2	83.4187	77.5303	1/10	83.4138	77.5306
C2	83.4145	77.5254	I2	83.4216	78.1346	1/100	83.4163	77.5335
C2	83.4145	77.5262	I2	83.4216	77.2340	1/100	83.4163	77.5335
C2	83.4350	77.5262	I2	83.4075	77.2340	1/100	83.4163	77.5335
D2	83.4210	77.5301	L2	83.4231	77.5288	1/1000	83.4238	77.5343
D2	83.4193	77.5303	L2	83.4231	77.5288	1/1000	83.4238	77.5335
D2	83.4193	77.5309	L2	83.4231	77.5449	1/1000	83.4190	77.5335
E2	83.4187	77.0454	M2	83.4203	77.5285	1/10000	83.4187	77.5249
E2	83.4352	77.5445	M2	83.4203	77.5267	1/10000	83.4187	77.5249
E2	83.4187	78.0201	M2	83.4203	77.5267	1/10000	83.4187	77.5249
F2	83.4237	77.5528	N2	83.4167	77.5254	1/100000	83.4206	77.5256
F2	83.4179	77.5108	N2	83.4167	77.5254	1/100000	83.4187	77.5249
F2	83.4238	77.5108	N2	83.4235	77.5254	1/100000	83.4206	77.5249

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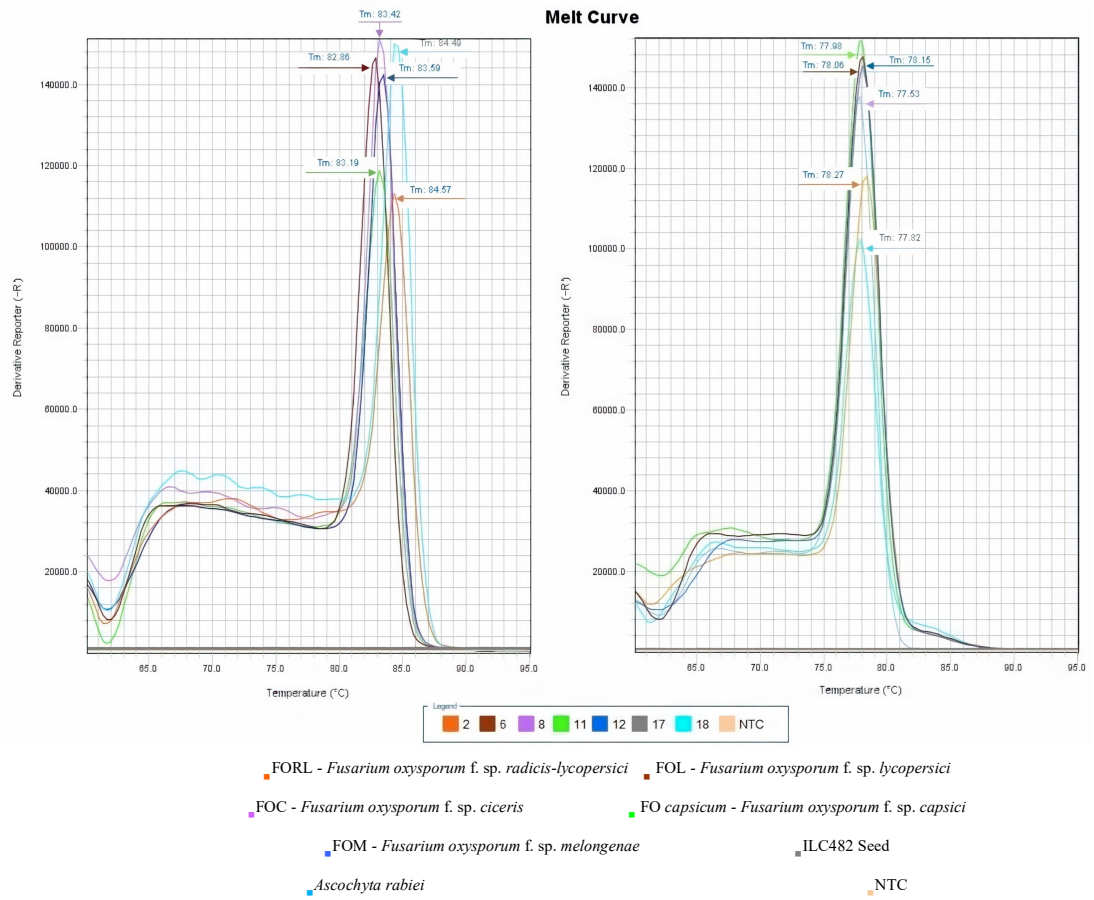

Figure 4. Melt Curve of infected different samples for H3 (a) and J5 (b) primers.

qPCR analysis and quantitative determination of Fusarium wilt agent in plants using the SYBR Green master mix. The quantification ability of designed primer pairs was tested on chickpea plants inoculated with FOC and quantity of pathogen was determined. Ct values of samples were placed on standard curve obtained from qPCR of positive control dilutions for both primers indicating precise quantification of fungal agent in inoculated plant samples. In addition to the study procedure, the accuracy of our dilution factor for positive controls was also determined when the amplification graphs were examined (Figure 5 and Figure 6). The threshold, known as a statistically significant signal level was drawn automatically by the device.

Quantitative amount, Ct and mean Ct values of growth chamber material were compared on the basis of primer pairs. The results suggest that the J5 primer pair separated the wilting form better than the yellowing from of FOC. The H3 primer pair identified the yellowing form better than the wilting form of FOC. Similarly, symptomatic distinction of yellowing and wilting forms was performed easily with the J5 primer pair than that of the H3 primer pair. One-Way ANOVA analysis together with the Tukey test was applied and confirmed a significant difference ($P< 0.05$) (Figure 7 and Table 4).

The FOC was determined 24 hours after inoculation with qPCR, however, disease symptoms were observed 3 days after inoculation. A reliability test identified that the J5 primer pair provided better detection

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Table 3. Melting temperature for quantification of *Fusarium oxysporum* f. sp. and *Ascochyta rabiei* by qPCR compared with *Fusarium oxysporum* f. sp. *ciceris* low virulent wilting form samples.

H3						J5					
Isolates	Code	Tm (°C)	Isolates	Code	Tm (°C)	Isolates	Code	Tm (°C)	Isolates	Code	Tm (°C)
	1	84.5748		11	83.1904		1	78.2678		11	77.9785
	1	84.5642	FO *capsicum*	11	83.1904		1	78.2678	FO *capsicum*	11	77.9785
	1	84.5455		11	83.1892		1	78.2653		11	77.9686
	2	84.5706		12	83.5886		2	78.2678		12	78.1499
FORL	2	84.5650		12	83.5935	FORL	2	78.2678		12	78.1371
	2	84.5493		12	83.5893		2	78.2653		12	78.1477
	3	84.5638		13	83.5893		3	78.2653		13	78.1497
	3	84.5661	FOM	13	83.5872		3	78.2671	FOM	13	78.1264
	3	84.5666		13	83.5872		3	78.2671		13	78.1264
	4	82.8500		14	83.5938		4	78.0571		14	78.1497
	4	82.8392		14	83.5871		4	78.0609		14	78.1454
	4	82.8465		14	83.5871		4	78.0616		14	78.1454
	5	82.8350		18	84.4033		5	78.0591		18	77.8168
FOL	5	82.8287		18	84.4038	FOL	5	78.0591		18	77.8204
	5	82.8492		18	84.3936		5	78.0644		18	77.8204
	6	82.8582		19	84.4033		6	78.0499		19	77.8198
	6	82.8582	*Ascochyta rabiei*	19	84.3954		6	78.0484	*Ascochyta rabiei*	19	77.8211
	6	82.8633		19	84.3954		6	78.0578		19	77.8211
Sezenbey Root Collar	8	83.4209		20	84.3854	Sezenbey Root Collar	8	77.5309		20	77.8159
	8	83.4175		20	84.3763		8	77.5309		20	77.8171
	8	83.4187		20	84.3763		8	77.5296		20	77.8219

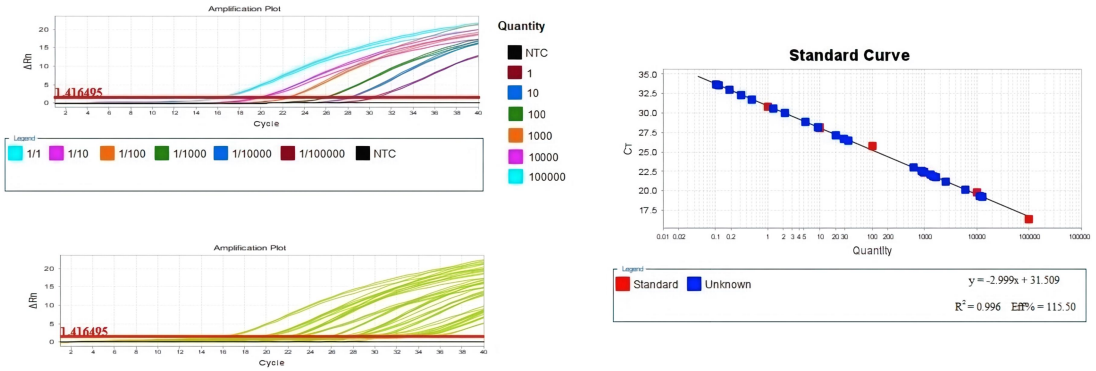

Figure 5. (a) Amplification curves of H3 primer set of different dilution level of cDNA from low virulent wilting form (positive control) (b) Amplification curve (Ct) and (c) Standard curve of H3 primer set from plant sample inoculated with low virulent wilting form on sampling days.

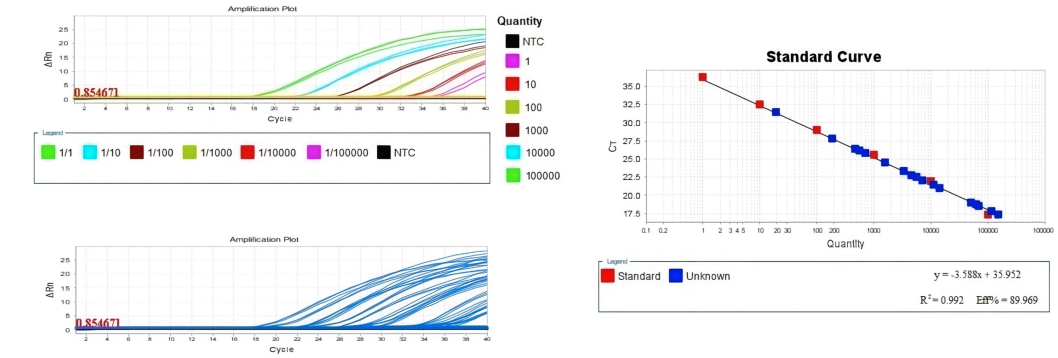

Figure 6. (a) Amplification curves of J5 primer set of different dilution level of cDNA from low virulent wilting form (positive control) (b)Amplification curve (Ct) and (c) Standard curve of J5 primer set from plant sample inoculated with low virulent wilting form on sampling days.

than H3 because it had higher specificity to FO cDNA. According to the statistical reliability test performed on the quantitation data determined by the primers, it was found that the J5 primer pair has higher reliability than the H3 primer pair [α_{H3}= 0.812 and α_{J5}= 0.836 ($\alpha \geq$ 0.80 means highly reliable)] (Table 5).

The Pearson's correlation test was applied on DSI% (Disease Severity Index) and Ct of both primers for every sampling day. High correlation ($P < 0.01$) between DSI% and Ct was observed. At the same time, high degree of correlation was observed between primer pairs ($r \geq 0.65$ means high correlation). Only negative correlation was observed, because the smaller Ct value means the higher the amount of pathogen fungal biomass (Table 6).

Considering the presence or absence of fungal agent in the seed, FOC was also detected in infected seed, root collar and root samples by culture-pathogenicity studies and qPCR assay. In addition, as a result of the study with other FO f. sp. and *A. rabiei*, it was determined that FOC was differentiated. In this respect, One-Way ANOVA analysis together with the Tukey test was applied on Ct results ($P < 0.05$) (Figure 8) of qPCR applied on cDNA from field and fungal pathogens other than FOC. A significant difference between groups was observed, and results of Tukey test defined the presence of a completely different group (e.g. e) based on Ct values. *A. rabiei*, which is an important disease agent in chickpea, was included in the study for evaluating the specificity of our primers and, as shown in

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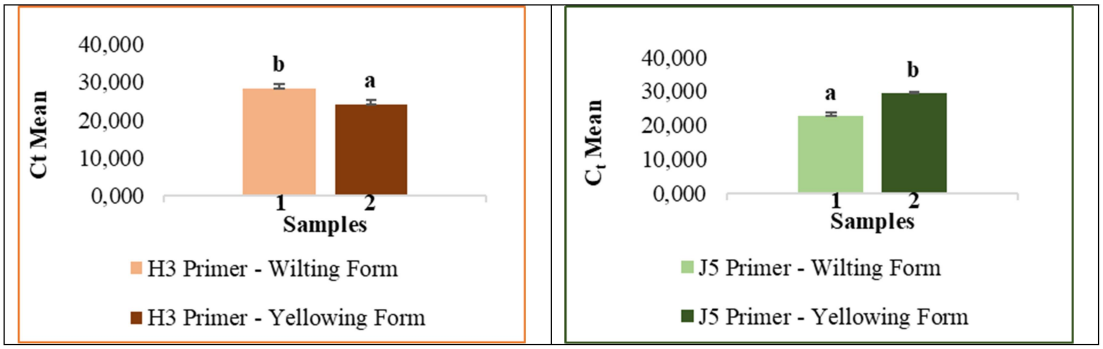

Figure 7. Graph of Ct of H3 (a) and J5 (b) primers according to infected samples with *Fusarium oxysporum* f. sp. *ciceris*.

Table 4. One-Way ANOVA test of quantitation amounts of *Fusarium oxysporum* f. sp. *ciceris* based on H3 and J5 primer pairs in qPCR.

Forms	H3 Quantification				Forms	J5 Quantification			
	Mean	df	F value	P value		Mean	df	F value	P value
Wilting	4389.2295[a]	2	1.094	0.342	Wilting	21838.1147[a]	2	6.667	0.003
Yellowing	2771.0198[a]				Yellowing	3326.2213[b]			

P< 0.05.

Table 5. Reliability test of primer pairs for quantification of *Fusarium oxysporum* f. sp. *ciceris* in qPCR.

	Cronbach's Alpha	N
H3 primer pair	0.812	30
J5 primer pair	0.836	30

Table 6. Pearson correlation test of H3 and J5 primer pairs on Ct and DSI% values.

N= 48		1	2	3
DSI% (1)	Pearson correlation	1.000		
	P	-		
H3 Ct (2)	Pearson correlation	−0.822**	1.000	
	P	0.000	-	
J5 Ct (3)	Pearson correlation	−0.647**	0.836**	1.000
	P	0.000	0.000	-

** P< 0.01-* P< 0.05.

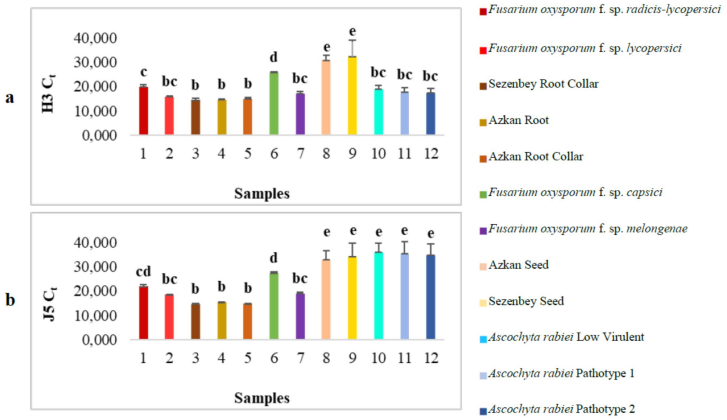

Figure 8. Differentiation graph of $Ct_{(H3)}$ (a) and $Ct_{(J5)}$ (b) primers for the other pathogenic fungi.

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Figure 8, it was not differentiated with the H3 primer.

A reliability test was applied on the quantitation data determined by the primer pairs, and both primer pairs exhibited high reliability [α_{H3}= 0.944 and α_{J5}= 0.995 ($\alpha \geq$ 0.80 means highly reliable)] (Table 7). Thus, it was identified that the J5 primer pair differentiates the ff. spp. of FO are better than the H3 primer pair. In addition, the H3 primer pair provides clear differentiation for FOC in seed.

Confidence of Quantitative Measurement

The concentrations of all total RNA samples were diluted to 100 ng. Standard curves were generated using 10-fold dilutions of cDNA ranging from 5 ng to 0.005 pg obtained from pure fungal culture (Figure 9). However, since the 0.005 pg dilution fell outside the standard curve, the detection limit was set to a dilution factor of 10^5 and used throughout the study. The regression equations for the standard curves were calculated using qPCR systems software (Figure 5, Figure 6 and Figure 9). When the R^2 values of the standard curves were analyzed, the R^2 values $\geq$ 0.98 provided good confidence in the experimental design (choosing, designing and productivity of appropriate primer pairs). One sample t-test ($P< 0.05$) was applied to values obtained from the studies to observe the difference between the obtained R^2 of both primers and the standard

Table 7. Reliability test of primer pairs used for differentiation of *Fusarium oxysporum* f. sp. in qPCR.

	Cronbach's Alpha	N
H3 primer pair	0.944	13
J5 primer pair	0.995	13

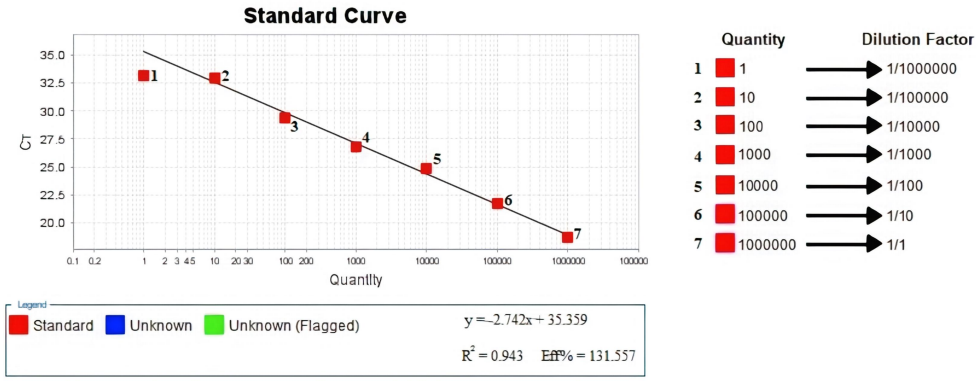

Figure 9. Standard curve graph according to dilution factor of obtained cDNA from 100 ng μL^{-1} total RNA.

Table 8. One sample t-test of R^2 values for quantification of *Fusarium oxysporum* f. sp. *ciceris*.

Groups				N	$\bar{X}$	SS	T	P
R^2 standard value (0.9945)	H3	Wilting	High Virulent	20	0.987	0.005	−1.719	0.102
			Low Virulent		0.996			
		Yellowing	High Virulent		0.990			
			Low Virulent		0.998			
	J5	Wilting	High Virulent	20	0.997	0.003	1.619	0.122
			Low Virulent		0.992			
		Yellowing	High Virulent		0.999			
			Low Virulent		0.994			

$P< 0.05$.

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R^2 value (Tables 8 and 9). When the results are examined, there is no statistically significant difference between the R^2 values obtained from the H3 and J5 primer pairs quantification on the basis of standard R^2 data ($t_{H3}(20)= -1.719$, $P> 0.05$; $t_{J5}(20)= 1.619$ $P> 0.05$ and $t_{H3}(76)= -0.951$, $P> 0.05$; $t_{J5}(76)= -1.846$, $P> 0.05$) (Tables 8 and 9). Values such as R^2, efficiency %, Ct and quantitative amounts were automatically calculated by the same software. The PCR efficiency was calculated using (Efficiency= $10^{(1/slope)}-1$) formula. A Pearson's correlation test was used to define relationships between pathogen cDNA quantity, efficiency (%), R^2 and Ct levels, as well as the relationships between cDNA quantities of different pathogens and samples, and efficiency (%), R^2 and Ct levels ($P< 0.01$ and $P< 0.05$) (Tables 10 and 11). The Pearson's correlation test results revealed statistically significant correlation between Cts and DNA quantities ($P< 0.01$ and $P< 0.05$), and the correlation coefficients were significantly different from zero ($rCt_{(H3)}= 0.541$; $rCt_{(J5)}= 0.650$ and $rCt_{(H3)}= 0.556$; $rCt_{(J5)}= 0.575$) (Tables 10 and 11).

DISCUSSION

Chickpea is the first cultivated edible grain legume, and it is an important nutrition source (Ladizinsky, 1975). Fusarium wilt disease, which has two different disease forms, is defined as the most "Destructive Disease" of chickpea in the world (Priyanka *et al*., 2021), and both forms of the disease (yellowing, wilting) were reported from Turkey (Kocalar *et al*., 2020). In addition to this, causal agent of chickpea Fusarium wilt has high pathogenic variability and can survive in the soil for up to 6 years as chlamydospore, making control and management of the disease more challenging (Desai *et al*., 2019). The present study demonstrated that qPCR is a powerful technique for precise determination and

Table 9. One sample t-test of R^2 values for quantification of other pathogenic fungi.

Groups				N	X̄	SS	T	P
R^2 standard value (0.9945)	H3	Wilting	High Virulent	76	0.996	0.002	−0.951	0.345
			Low Virulent		0.992			
		Yellowing	High Virulent		0.997			
			Low Virulent		0.992			
	J5	Wilting	High Virulent	76	0.998	0.002	−1.846	0.069
			Low Virulent		0.993			
		Yellowing	High Virulent		0.992			
			Low Virulent		0.993			

$P< 0.05$.

Table 10. Pearson correlation test of Ct, R^2 and efficiency (%) in comparison with *Fusarium oxysporum* f. sp. *ciceris* quantity of infected plants.

		H3 primer pair (N= 48)				J5 primer pair (N= 48)			
Parameter		1	2	3	4	1	2	3	4
Quantity (1)	Pearson Correlation	1.000				1.000			
	P	-				-			
Ct (2)	Pearson Correlation	−0.541**	1.000			−0.650**	1.000		
	P	0.000	-			0.000	-		
R^2 (3)	Pearson Correlation	0.313*	0.129	1.000		0.351*	0.566**	1.000	
	P	0.030	0.384	-		0.015	0.000	-	
Efficiency (%) (4)	Pearson Correlation	0.304*	0.053	0.962**	1.000	0.342*	0.567**	0.945**	1.000
	P	0.036	0.722	0.000	-	0.0170	0.000	0.000	-

** $P< 0.01$ - * $P< 0.05$.

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Table 11. Pearson correlation test of Ct, R^2 and efficiency (%) in comparison with other pathogenic fungi quantity.

		H3 primer pair (N= 48)				J5 primer pair (N= 48)			
Parameter		1	2	3	4	1	2	3	4
Quantity (1)	Pearson Correlation	1.000				1.000			
	P	-				-			
Ct (2)	Pearson Correlation	−0.556**	1.000			−0.575**	1.000		
	P	0.000	-			0.000	-		
R^2 (3)	Pearson Correlation	0.300*	0.194	1.000		0.300*	0.015	1.000	
	P	0.011	0.868	-		0.018	0.868	-	
Efficiency (%) (4)	Pearson Correlation	0.261*	0.067	0.512**	1.000	0.342*	0.062	0.512**	1.000
	P	0.027	0.568	0.000	-	0.0170	0.822	0.000	-

** P< 0.01 - * P< 0.05.

quantification of FOC, which severely diminishes chickpea production all over the world. In addition, data from this device can be useful for DM.

Traditional identification of fungi based on morphological and microscopical characteristic may not always accurately distinguish species complex; furthermore, none of the available techniques allow to quantify pathogens from infected plants (Pollard and Okubara, 2019; Priyadarshini *et al*., 2021). Because they are more specific, sensitive, and accurate than traditional methods, molecular and biotechnological techniques help to avoid many disadvantages associated with traditional identification methods and improve pathogen detection under various conditions. Furthermore, qPCR offers potentially quick, credible, low-cost and a reproducible analysis method (Naroei and Salari, 2015; Hubballi *et al*., 2022).

Applications of qPCR offers some opportunities for characterization of phytopathogenic and antagonistic fungi, as well as culture-independent ability of identification that distinguishes it from many other methods (Schena *et al*., 2004). The qPCR technique, which is generally applied for the determination of gene expression levels, was used for precise quantification of fungal biomass (Demirel, 2019). This technique also allows for the quantification of disease in plants that are exhibiting disease symptoms, in asymptomatic plants or directly from seed. In the current study, FOC was instantly detected after the first day of inoculation by qPCR assay, but disease symptoms were not observed until the third day in growth chamber. We support de Sousa *et al*. (2015) and Pollard and Okubara's (2019) views with the result of the current study. That is, we suggest using qPCR devices with good sensitivity, even in small quantities, for detection and quantification of FOC in seeds. In addition to quantification studies, causal agent inducing wilting and yellowing in chickpea was differentiated from other pathogenic fungi by this method.

Lees *et al*. (2002) explained that RNA should be chosen instead of DNA in qPCR as the target molecule because it could never interfere with the analysis performed, and then it must be reverse transcribed into cDNA. On the other hand, quality of the purity of target RNA (28S-18S examination) is a significant issue (Bradford *et al*., 2005). In this study, as opposed to the studies of de Sousa *et al*. (2015) and Pollard and Okubara (2019), template for RT-qPCR was obtained by cDNA conversions of total RNAs isolated from root collar of infected plant samples. The most important feature of this qPCR technology is its relatively low detection threshold at nucleic acid concentration as low as $5\text{-}5x10^{-5}$ ng. The results we obtained from the seeds support this unique feature. In other words, even if the seeds contain a small amount of infection, it is clear evidence that these devices, due to their sensitivity, precisely determine them.

Fraga *et al*. (2014) stated that qPCR reactions have three different steps with unique properties (exponential, linear and

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plateau phases). Therefore, at this point, experimental design is of great importance (Taylor *et al*., 2019). For such reasons, each step of the study should be designed with careful consideration of the variable factors, and the obtained results should be statistically analyzed separately to determine reliability, reproducibility, specificity, etc. Pathogen-specific primer pairs (H3 and J5) were designed for quantification of the fungal pathogen causing chickpea wilting and yellowing. The H3 and J5 gene regions were chosen for use in qPCR studies, because they are protected throughout the evolutionary process and are overexpressed during pathogen attack (Thatcher *et al*., 1994; Jeong *et al*., 2005). With the study of pathogen quantification, it was detected that both specific primers were able to identify the amount of the pathogen from the first day after inoculation (the early stages of the disease). In addition, with the reliability test for studies, it was determined that the J5 primer pair was more reliable. Results of One-Way ANOVA analysis suggests that the H3 specific primer pair effectively determined yellowing form of the disease, while J5 specific primer pair determined the wilting form more efficiently. It was also determined that the H3 primer pair provided a better separation in the seed samples (One-Way ANOVA). According to the correlation test performed with the disease severity and with the Ct's of the primer pairs used in qPCR, the Ct of the J5 primer pair exhibited stronger correlation with the disease severity than the H3 primer pair.

Çolak and Bicici (2013) specifically recommend PCR-based molecular techniques for the rapid and reliable identification of f. sp. and races of FO. In addition to all this, similar to *F. oxysporum* f. sp. *lycopersici*, the morphological differentiation of wilting and yellowing forms caused by FOC is very difficult under *in*-vitro conditions. These forms are sometimes confused with each other and lead to false-positive results. According to the One-Way ANOVA analysis performed, the yellowing and wilting forms could be differentiated from each other depending on the quantitation amounts and Ct values obtained by using the J5 primer pair. Besides, when the results of the H3 primer pair were examined, it was determined that only Ct values were significant for differentiation.

The threshold Cycle (Ct) or the noise level is known as a measure of the relative target concentration in the result of the qPCR reaction, and involves comparing the Ct values of assay samples with the defined standard quantity plotted on a standard curve (Mahboudi *et al*., 2018). For this aim, serial dilutions of the positive control samples at known concentrations are used to create a standard curve, similar to the study of de Sousa *et al*. (2015). Thus, the qPCR permits the finding out of the relative quantities of unknown samples depending on their Ct values. Additionally, each sample should be studied in three replications to determine whether there are any inconsistent results, to increase both the reliability and specificity of primer pairs, and to minimize the chance of wrong results. Within the scope of studies carried out for this purpose, the results were similar to those of Mirmajlessi *et al*. (2015) as it is a device with high reproducibility (sensitivity), rapid detection and low risk of contamination, as well as Mahboudi *et al*. (2018) with efficiency, Ct examination, and a 3-replicate experiment set up. In addition, when applying the SYBR Green assay a melting curve analysis has been recommended by experts to test specificity of primer used for the qPCR assays. SYBR Green was used in the study, and the results of a melting curve analysis indicate that the designed primers did not formed any primer dimers.

Absolute quantification studies are based on conceptually quite simple and accurate calculations of template in qPCR. The mean standard curve slope should be within a certain value range limit (110%≤ Efficiency (E)≤ 90% for −3.1≤ Slope (S)≤ −3.6). Furthermore, the value of R^2 in the standard curve must be ≥ 0.98 due to its association with reliability of results. Although the

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methodologies are the same, efficiency and similar factors play a very important role in proportional differentiations as they can vary from method to method. In addition, "the data may even differ from device to device" because the sensitivity and accuracy of the qPCR device depend on the detector system (Mahboudi *et al*., 2018). The use of the best method in the relative examination for high efficiency is very important in terms of the efficiency and reproducibility of the method in the experimental setup. We offer a reliable, realistic view of productivity within the framework of statistical results of our studies.

CONCLUSIONS

Quantification of chickpea Fusarium wilt agent, FOC, was successfully performed using species-specific H3 and J5 primer pairs. At the same time, this relatively new application presents an effective and real time monitoring technique, even in the presence of a low amount of the fungal pathogen in plant tissues. qPCR detected the disease agent with 0.05 pg of cDNA per reaction in the assays, distinguished the targeted fungal agent from other fungal pathogens, and identified the pathogen even in the seeds with a statistically significant difference ($P< 0.05$). The results obtained in this study provide a method that allows the direct, faster and more accurate determination of FOC by eliminating the problems encountered in traditional methods. The results of the current study will facilitate understanding of the interaction between disease and plant by examining the inoculum load in seed and plant. The protocols applied during investigations could decrease/limit the spread of pathogens to new areas in terms of DM and BC. Therefore, effective management methods for rapid and reliable inspection of the areas suffering from FOC and the preventive actions against disease dispersal can be established.

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ارزیابی کمی پژمردگی فوزاریوم نخود

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چکیده

گونه *Fusarium oxysporum* f. sp. *ciceris* (FOC) عامل پژمردگی فوزاریوم است که یک بیماری مخرب و گسترده نخود می باشد. شناسایی و تشخیص سریع و دقیق پاتوژن های گیاهی برای راهبرد (استراتژی) های مدیریت بیماری (DM) با اقدامات مناسب ضروری است. این پژوهش با هدف تعیین کمی FOC با استفاده از تکنیک واکنش زنجیره‌ای پلیمرازدر زمان واقعی (qPCR) با جفت آغازگرهای خاص [Histone (H3) و Ribosomal (J5)] در دانه، ریشه و یقه ریشه و تمایز آن از دیگر قارچ های بیماری زا [*Fusarium oxysporum* formae speciales (FO f. sp.) و *Ascocyhta rabiei*] انجام شد. کل RNA

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های جدا شده، به cDNA تبدیل شدند (حد۰/۰۵ pg/rxn- ۵ ng/rxn)و به عنوان الگو برای بررسی‌های qPCR مورد استفاده قرار گرفت. FOC در نمونه های گیاهی از روز اول پس از تلقیح شناسایی شد. FOC در نمونه‌های ریشه، یقه ریشه و بذر شناسایی شد و با استفاده از روش qPCR از سایر قارچ‌های بیماری‌زا متمایز شد. منحنی های ذوب، که در آن هیچ دایمر آغازگر(primer dimers) و مکمل غیر اختصاصی مشاهده نشد، یک اوج (پیک) واحد را ارائه کرد. تعیین کمیت با استفاده از جفت پرایمرهای خاص H3 و J5 با موفقیت انجام شد (p<۰/۰۵)، و FOC با پرایمر J5 از سایر قارچ‌های بیماری‌زا متمایز شد (p<۰/۰۵). نتایج این بررسی‌ها ممکن است موید و حامی توسعه روش‌های بیوشیمیایی و مولکولی جدید باشد که امکان تعیین مستقیم، سریع‌تر و دقیق‌تر عوامل بیماری‌زا را فراهم می‌کند. بنابراین، ما را قادر می سازد تا خسارت‌های ناشی از بیماری ها و هزینه های مدیریت بیماری را کاهش دهیم.

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