The use of Real-Time PCR Assay for Screening Chickpea Genotypes Resistant to Ascochyta Rabiei

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Abstract

Ascochyta blight, caused by *Ascochyta rabiei* is a devastating disease of chickpea worldwide. Breeding for host resistance is an efficient means to reduce the damage by this pathogen. This study evaluated the utility of Real-time polymerase chain reaction (PCR) assay for screening chickpea genotypes for resistant to blight disease. Eight days after inoculation, the resistance level of 84 chickpea genotypes was quantified by Real-time PCR technique using a standard curve constructed by amplifying different known amounts of target DNA and compared with disease scores based on visual assessment. A significant variation was statistically found among chickpea genotypes with respect to disease reactions. The quantity of target DNA in infected samples varied from 0.004–83.37 ng. The results demonstrated a strong relationship between visual scoring of disease severity and quantification of the target DNA in chickpea genotypes. Tüb-35, Tüb-47, Tüb-26, Tüb-82, Tüb-65 and Tüb-69 genotypes were found highly resistant to Ascochyta blight based on the results of both assays utilized for screening chickpea genotypes for resistance. The real-time PCR assay could be used for quantifying disease progression in plant tissues and screening chickpea genotypes as a potential alternative to visual scoring in plant resistance breeding programs.

Introduction

Chickpea (*Cicer arietinum* L.) is an important food legume crop with an annual global production of about 14.78 million tons [1]. Turkey is a major chickpea producing country with harvested area of 392 673 ha producing of 470 000 t [1]. Among biotic stresses limiting chickpea production, Ascochyta blight caused by the fungus *Ascochyta rabiei* is the most destructive foliar disease affecting chickpea cultivation worldwide. The fungus can infect all above ground parts of the plant and survives on infested crop residue and seed. The fungus is extremely destructive and significantly affects the yield and quality of chickpea. The yield losses can reach up to 100% under favorable conditions [2]. The presence of a sexual phase (*Didymella rabiei*) in the life cycle of the pathogen leads to high level of variability in aggressiveness within the pathogen populations [2]. Several pathotypes of the fungus have been determined based on the variations in host-pathogen interactions in many countries [3, 4, 5]. Pathotypes 1 and 3 are widely available in chickpea production areas of Turkey [6].

Fungicide applications are used to control Ascochyta blight in many areas of the world [2, 7]. However, the efficacy of fungicides treatments used for controlling this disease varies depending on host resistance, disease pressure, fungicide efficacy, and environmental concerns [8, 9]. Foliar sprays are sometimes impractical and uneconomical due to the application of foliar spraying several times to control the disease [10, 8]. Thus, the use of durable resistant cultivars to control Ascochyta blight is suggested as the most effective and convenient approach [9, 11, 12].

Many comprehensive studies have been carried out for elucidating the mechanisms underlying resistance to Ascochyta blight by using traditional and biotechnological techniques [12, 13, 14]. Sources of partial resistance have been identified in some chickpea germplasms and employed in breeding programmes for

resistance to Ascochyta blight [2, 15, 16]. However, breakdown of resistant sources to the disease can occur rapidly under natural conditions as new pathogenic variants arise within the pathogen populations [5, 11, 17]. This requires continuously the identification of novel genetic sources of host disease resistance and the incorporation of these into chickpea cultivars with adaptation to different agro-ecosystem. To date, a number of screening methods have been reported and routinely employed for screening chickpea genotypes to Ascochyta blight in different environment conditions [2, 11]. However, the efficacy of resistance screening methods varies depending on interactions between pathogen and host genetics and environmental conditions [11]. Thus, more accurate, reproducible, and consistent approaches are needed for disease assessments in resistance breeding studies [18]. Real-time PCR is a reliable and sensitive tool for detecting and quantifying the target DNA [19, 20]. The method has been extensively used for accurate monitoring of disease progression in infected plant tissues as an optional method to visual scoring of disease severity [21, 22]. Quantitative analysis of *A. rabiei* infection in chickpea has been developed by Bayraktar et al. [23]. This study evaluated the efficacy of Real-time PCR technique for selecting resistant breeding material to Ascochyta blight.

Materials And Methods

Plant and pathogen materials

Chickpea genotypes were selected by taking into account yield advantages of candidate genotypes according to the results of field yield trials conducted at the research areas of the Central Research Institute for Field Crops in Ankara, Turkey. Also, the resistance cultivar-ILC 482 and the susceptible cultivar-Sarı-98 were included in the study for confirmation of disease reaction in chickpea genotypes. Chickpea seeds were treated for 2 min with 1% NaOCl, washed three times with tap water, and planted in pots comprising the pasteurized soil mixture (sand, soil and manure vol/vol). They were then incubated in the chamber at 23°C under fluorescent growth lights with 12-h photoperiod of for 15 days.

Ascochyta rabiei isolate, Asc30, belonging to pathotype 1 was provided from the culture collection of Ankara University, Department of Plant Protection Turkey. The isolate was grown on CSMDA medium (chickpea seed meal: 40 g, dextrose: 20 g and agar: 18 g in 1 L distilled water) for 15 days at 20–23°C. Fungal mycelia for DNA extraction was scraped off of the surface with a sterile spatula and placed in an Eppendorf tube. For plant inoculation, spores were collected by adding 20 mL of sterile distilled water to each plate and scraping the agar surface using a glass spatula. The spore concentration was counted in a hemacytometer and standardized a final concentration of about 1.5×10^5 conidia ⁄ mL.

Inoculation and disease assessments

Detached leaf screening technique was used to evaluate chickpea genotypes for resistance to *A. rabiei* [24, 25]. The second and third leaflets from the top of 84 genotypes, grown as mentioned above were excised, surface-disinfested and placed with the bottom side facing upwards on sterile distilled water in a Petri dish. Spore suspensions containing 1.5×10^5 spores ⁄ mL were applied in droplets of 5 µl on the

upper surfaces of the leaflets and, the plates were then kept in the dark for 8 days at 20°C. Disease severity was determined from the estimated size of lesions on leaflet based on 0–5 scale, where 0 indicated no lesion, 1, 2, 3, 4, and 5 indicated 10, 25, 50, 75 and 100% of leaflet area affected. Chickpea genotype was considered as highly resistant, resistant, moderately susceptible, or highly susceptible if the disease severity value was 0–10%, 11–29%, 30–49% or > 50%, respectively. Three plates for each genotype were evaluated and each plate contained ten leaflets. Control detached leaflets were inoculated with sterile distilled water in the same manner. Data of disease severity among chickpea genotypes was analyzed using MSTAT-C (Michigan State Univ., USA) computer program and the significant differences were separated using LSD test (Least Significant Difference test, P = 0.05). Immediately after assessment, the leaflets collected from each plate were transferred to 2 ml Eppendorf tube and kept at -80 ^{O}C until required for extraction.

Extraction of DNA from pure culture and plant material

Total DNA was purified from mycelium and from detached leaflets using Thermo Scientific-GeneJet Plant Genomic DNA Purification Mini Extraction kit. DNA concentration and quality was estimated according to the values of A260 nm/A280 nm using a NanoDrop-1000 spectrophotometer. DNA samples were diluted to 20 ng/µL and kept at -20°C for later use.

Quantification of the target DNA

qPCR analysis was carried out in LightCycler 96 Real-Time PCR System (Roche Diagnostics) using specific primers (HEF1/2, CTTATGGGCTCCGGTCCA AG/GCTTCCTGTGGTTGTCAG) for Eukaryotic elongation factor 1 EF-1 alpha gene (23). PCR mixture was prepared in a total volume of 20 µL using 2× FastStart Essential Green Master Mix (Roche Diagnostics), 0.25 µM of each primer, 5 µL of template DNA (20 ng/µL). Cycling conditions were 95°C for 10 minutes, then 45 cycles of 95°C for 10 seconds, 63°C for 10 seconds and 72°C for 5 seconds. After each cycle, specific amplification was verified by melting curve analysis. A melt curve was generated by heating from 65°C to 95°C at 0.5°C/step and measuring the fluorescence at each step. The efficiency and sensitivity of PCR amplification was analyzed by using calibration curves constructed from a series of the 10-fold diluted genomic DNA (100 ng − 0.01 pg) from pure *A. rabiei* culture with chickpea DNA of concentration 20 ng/ µL. The relationship between the values of visual scoring and the amount of target DNA in different chickpea genotypes was compared using Minitab 17 statistical software at the $P \leq 0.05$ level.

Results And Discussion

Breeding host resistance to *Ascochyta rabiei*, a destructive pathogen of chickpea worldwide is considered the most practical, effective and environmentally friendly method [11]. However, resistance breeding to this disease can be time-consuming and challenging because of the complex nature of host resistance, the evolving of continuously new pathotypes within the pathogen populations and the breakdown of resistance in commercially available cultivars [11, 26]. Comprehensive breeding studies by different research institutions in many countries have been continuously conducted for identification of new

resistance sources and screening breeding materials [18, 26, 27, 28]. The reliable and repeatable screening techniques are essential for the identification and screening of chickpea genotypes resistant to Ascochyta blight. Several assays developed for screening chickpea genotypes are now widely used [11]. However, these techniques have serious restrictions, especially the visual assessment of disease symptoms may show significant variations both between researchers and between different assessments of the same researcher [11, 18, 29]. In a previous study, we observed a strong relationship between disease assessment and quantification of pathogen infection in resistant and susceptible cultivars based on a quantitative real-time PCR assay [23]. Our results indicated that this approach may be a useful tool for the evaluation of breeding material for disease resistance during initial phases of infection as an optional approach to visual scoring and disease management. In this study, some chickpea genotypes were evaluated for the level of resistance to Ascochyta blight by detached leaf inoculation and quantitative real-time PCR assays.

Eighty-four chickpea genotypes selected from field yield trials were inoculated with detached leaflet method, and eight days after inoculation, leaflets were scored for disease severity. Significant variations were statistically found among disease severity of chickpea genotypes (LSD: 13.2, P: 0.05), ranging from 0–95.24% (Table 1, Fig. 1). The highest disease severity was observed in Tüb-108 genotype, following by Tüb-72 and Tüb-67 chickpea genotypes with disease severity ratings of %72.81 and %72.38, respectively. The lowest disease severity was determined in the genotypes of Tüb-47, Tüb-69, Tüb-26, Tüb-44, Tüb-22, Tüb-51 and Tüb-82 with a disease reaction of < 2%, respectively, while seven genotypes had disease severities of 2%. Twenty-four genotypes were resistant and forty-four were highly resistant, while sixteen genotypes were found susceptible or highly susceptible. Similar work was carried out by Chaudhry and Muhammad [30], who screened 867 samples to Ascochyta blight. 119 chickpea samples were susceptible, while 60 samples were found to be resistant. Toker and Cancı [31], screening 41 genotypes found 7 genotypes to be resistant. Akalın et al. [32], screening 50 chickpea genotypes to Ascochyta blight in Turkey, found that 5 genotypes were immune, 5 genotypes highly resistant, and 19 genotypes resistant or moderately resistant. The other 21 genotypes were regarded as tolerant or susceptible. Sahi et al. [33] evaluated 52 lines and indicated that three lines were resistant, 30 moderately resistant. Also, 12 and 3 lines exhibited susceptible and moderately susceptible reaction, respectively. Similar studies on the screening of chickpea materials for resistance to *Ascochyta rabiei* were performed by many researchers [28, 34, 35].

Table 1
Disease reaction of chickpea genotypes inoculated with *Ascochyta rabiei*

Chickpea genotypes	Disease severity %*	Disease reaction	Chickpea genotypes	Disease severity %	Disease reaction
Tüb-01	23.33	R	Tüb-48	14.67	R
Tüb-02	56.19	HS	Tüb-49	10.67	R
Tüb-03	16.67	R	Tüb-50	15.33	R
Tüb-04	24.67	R	Tüb-51	1.33	HR
Tüb-05	8	HR	Tüb-52	21.9	R
Tüb-06	8.67	HR	Tüb-53	5.33	HR
Tüb-07	8.67	HR	Tüb-54	45.24	MS
Tüb-08	10	HR	Tüb-55	2.67	HR
Tüb-09	11.33	R	Tüb-56	2.67	HR
Tüb-10	8.67	HR	Tüb-57	6.67	HR
Tüb-11	2	HR	Tüb-58	28	R
Tüb-12	2	HR	Tüb-59	22	R
Tüb-13	6	HR	Tüb-60	16.67	R
Tüb-14	8	HR	Tüb-61	9.33	HR
Tüb-15	33.33	MS	Tüb-62	59.05	HS
Tüb-16	4	HR	Tüb-63	26.67	R
Tüb-19	4.67	HR	Tüb-64	22.86	R
Tüb-20	10.67	R	Tüb-65	2	HR
Tüb-21	2	HR	Tüb-67	72.38	HS
Tüb-22	1.33	HR	Tüb-68	37.14	MS
Tüb-23	2.67	HR	Tüb-69	0	HR
Tüb-24	4	HR	Tüb-70	2.67	HR
Tüb-25	2	HR	Tüb-71	14	R
Tüb-26	0.67	HR	Tüb-72	72.81	HS

*Disease severity was assessed with 0–5 scale (0: no lesion. 1: 10%; 2: 25%; 3: 50%. 4: 75% and 5: 100% of leaflet areas affected). Highly resistant (HR): 0–10%. Resistant (R): 11–29%. Moderately susceptible (MS): 30–49% and Highly susceptible (HS): >50%. Data are means of three replicates. Means compared with the least significant difference (LSD) (P = 0.05)

Chickpea genotypes	Disease severity %*	Disease reaction	Chickpea genotypes	Disease severity %	Disease reaction
Tüb-27	16.67	R	Tüb-76	21.9	R
Tüb-28	9.33	HR	Tüb-78	34.29	MS
Tüb-29	5.33	HR	Tüb-79	10	HR
Tüb-30	6	HR	Tüb-82	1.43	HR
Tüb-31	12	R	Tüb-84	44.05	MS
Tüb-33	14	R	Tüb-86	39.17	MS
Tüb-35	2	HR	Tüb-87	58.57	HS
Tüb-36	5.33	HR	Tüb-93	37.14	MS
Tüb-37	3.33	HR	Tüb-96	4	HR
Tüb-38	6.67	HR	Tüb-97	24	R
Tüb-39	4.67	HR	Tüb-100	11.33	R
Tüb-40	51.7	HS	Tüb-105	65.71	HS
Tüb-41	14.67	R	Tüb-108	95.24	HS
Tüb-42	8.67	HR	Tüb-114	4	HR
Tüb-43	2	HR	Tüb-119	29.52	R
Tüb-44	0.67	HR	Tüb-121	23.33	R
Tüb-45	4	HR	Tüb-124	46	MS
Tüb-46	4.67	HR	ILC482	9.33	HR
Tüb-47	0	HR	SARI	62.67	HS
LSD (P = 0.05):	13.02				

*Disease severity was assessed with 0–5 scale (0: no lesion. 1: 10%; 2: 25%; 3: 50%. 4: 75% and 5: 100% of leaflet areas affected). Highly resistant (HR): 0–10%. Resistant (R): 11–29%. Moderately susceptible (MS): 30–49% and Highly susceptible (HS): >50%. Data are means of three replicates. Means compared with the least significant difference (LSD) (P = 0.05)

Disease reaction of the genotypes evaluated to Ascochyta blight was also quantified by using a standard curves constructed by amplifying known amounts of target DNA by real-time PCR method developed in a previous study [23]. The primer pairs HEF1/2 amplified a PCR fragment of 82 bp in size from plant materials infected with *A. rabiei*. The slope and intercept value of the regression line for standards was 3.36 and 23.53, respectively, and the r^2 value was 1.00. The presence of a single melt peak confirmed the

amplification of a specific product with a dissociation temperature of 83°C. The assay showed significant variation among chickpea genotypes, and the amount of the target DNA in highly resistant genotypes was predominantly less than in more susceptible genotypes (Fig. 1). The quantity of pathogen DNA in the inoculated leaflet samples changed from 0.004–83.37 ng. The amount of target DNA quantified in cultivar ILC 482, highly resistant to Ascochyta blight was 1.87 ng, while DNA quantity in highly susceptible cultivar Sarı-98 was found as 43.07 ng. The results showed a highly correlation (r = 0.82) between the quantities of pathogen DNA and the levels of disease severity in chickpea genotypes. Among the chickpea genotypes tested, Tüb-35, Tüb-47, Tüb-26, Tüb-82, Tüb-65, Tüb-14, Tüb-16 and Tüb-69 genotypes with DNA quantities of ≤ 0.1 ng were classified as the most resistant to Ascochyta blight, while Tüb-105, Tüb-72, Tüb-67 and Tüb-108 genotypes were highly susceptible based on the quantification of the target DNA in leaflet tissues, respectively. Also, the average DNA quantities in highly susceptible, moderately susceptible, resistant and highly resistant genotypes exhibited significant differences (P ≤ 0.05). The average quantity of pathogen DNA in chickpea genotypes, exhibiting highly resistant response on visual disease scoring was 1.0 ng, while the average DNA quantity in chickpea genotypes regarded as resistant was determined as 5.37 ng. The average DNA quantity in moderately susceptible and highly susceptible genotypes was found to be 11.08 and 46.85 ng, respectively. Similarly, many studies on the assessments of the resistance levels of different host cultivars or genotypes by using quantitative real time PCR assay have been performed [21; 22]. Brouwer et al. [36] reported that the quantitative-real time PCR assay can be used for quick and reliable assessment of the susceptibility of Arabidopsis genotypes to different fungal pathogens. Similar work was conducted by Jimenaz et al. [37], who assessed the potential uses of qPCR for differentiating susceptible from resistant chickpea reactions to Fusarium wilt. A strong correlation (r = 0.87) between disease assessment and DNA quantity, suggested that qPCR assay may be used for screening resistance chickpea germplasm to Fusarium wilt. Daniëls et al. [38] which developed *Venturia inaequalis*-specific real-time PCR assay to analyze resistance of apple cultivars, found a significant correlation between the amount of DNA of *Venturia inaequalis* quantified in apple leaves and host resistance. They indicated this assay was a more objective and sensitive method than visual disease assessments to evaluate host resistance of apple cultivars. Leiminger et al. [39] evaluated relationship between visual disease assessments and DNA levels of *Alternaria solani* and *A. alternata* in potato leaves by quantitative PCR. They found a closely correlation (r = 0.71) between DNA quantities and the ratio of necrotic area caused by *A. solani* in agreement with the results of this study.

This study demonstrated the utility of real-time PCR assay for quick and reliable assessment of disease severity on chickpea genotypes to Ascochyta blight. A strong correlation was observed between DNA quantities and visual disease scoring in different chickpea genotypes. Tüb-35, Tüb-47, Tüb-26, Tüb-82, Tüb-65 and Tüb-69 genotypes were classified as resistant to Ascochyta blight based on both the levels of disease severity and the quantification of target DNA in infected plant tissues. These genotypes should be considered in detail in breeding studies as sources of resistance to Ascochyta blight. This assay could be used for selecting breeding material during initial phases of disease infection as an optional approach to visual score by quantifying the pathogen presence in host plant.

Declarations

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Authors' contributions

G. Özer and G. Palacıoğlu performed the experiment and drafted the manuscript. H. Bayraktar supervised the experiment, and revised of manuscript. A. Aydoğan provided the study materials. All authors read and approved the final manuscript.

Data availability statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

Consent to Participate This article does not contain any studies with human participants or animal models performed by any of the authors.

Consent to Publish The authors declare that they consent for publication of this study in *Molecular Biology Reports*.

Compliance with Ethical Standards Not applicable.

Ethical approval Not applicable.

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Figures

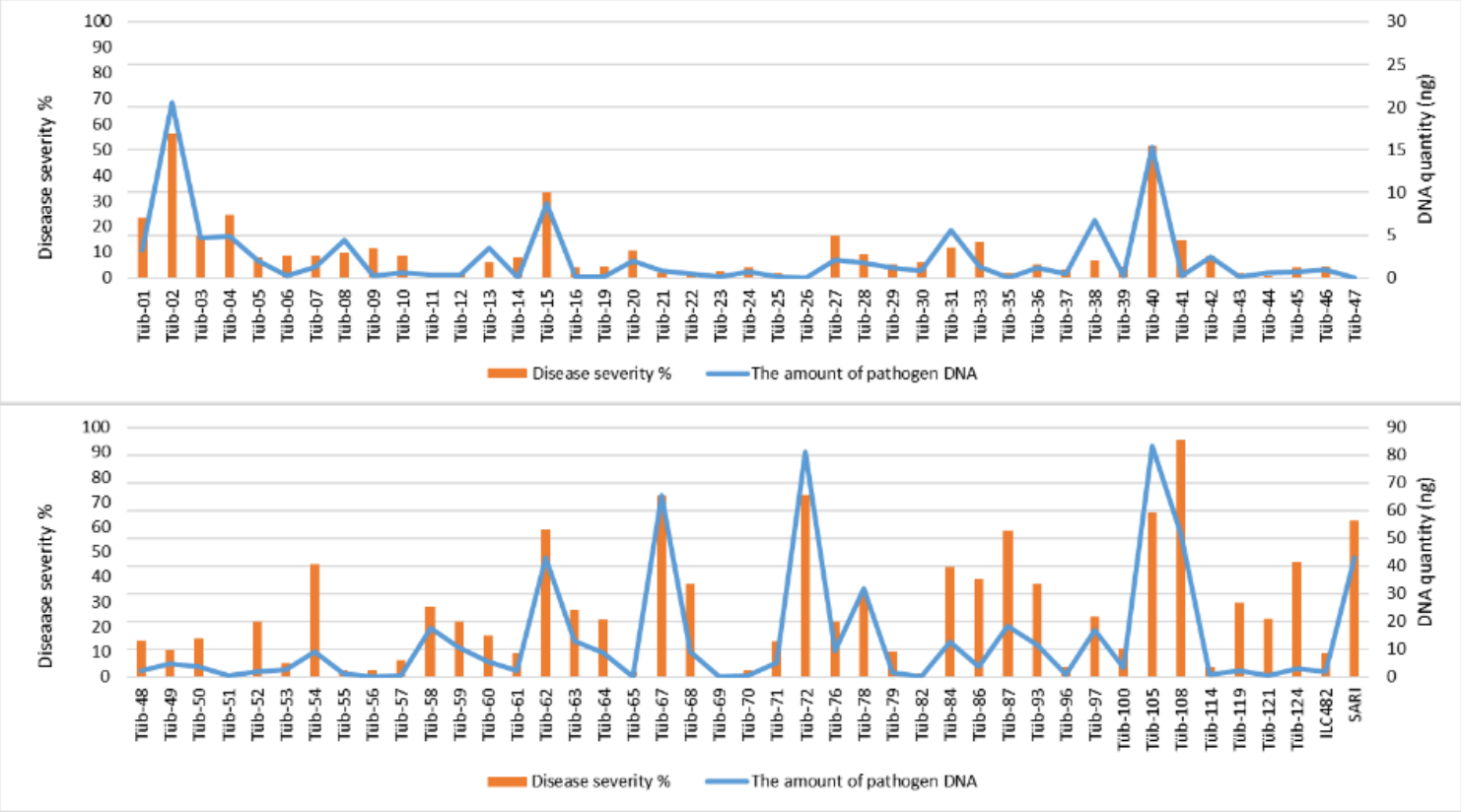

Figure 1

Comparison between the visual assessments of disease severity and the quantification of the target DNA in chickpea genotypes infected with Ascochyta rabiei