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Changes in Cytochrome *b* Gene Expression in *Cochliobolus sativus* Induced by Triadimefon, a Triazole Fungicide

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Abstract

Introduction: Spot blotch (SB), caused by *Cochliobolus sativus*, is most effectively managed using fungicide applications, including triadimefon (TDM) a triazole compound. *C. sativus* posses a great concern as it might develop resistance against fungicides like TDM due to its high genetic variability, short life cycle and abundant inoculum production. Therefore, to better understand the mechanisms of TDM resistance initiated by *C. sativus*, changes in cytochrome *b* (*cytb*) gene in virulent and avirulent pathotypes were evaluated at early time points of TDM treatments.

Materials and Methods: *C. sativus* sensitivity to TDM was determined by measuring radial growth of each pathotype on PDA plates. Additionally, RNA was isolated from mycelia of each pathotype at 24, 48, 72 and 96 hours post fungicide treatments and used for cDNA synthesis. *Cytb* was verified using quantitative reverse transcriptase PCR (qRT-PCR).

Results: Data showed that the maximum mycelial growth inhibition by 50% (EC50) for both pathotypes was recorded 48 hours at 0.25 µg mL^{-1} TDM treatment. The qRT-PCR revealed that *cytb* expression increased in both virulent and avirulent pathotypes at 24 hours post TDM treatments in comparison with non-treated controls. The most outstanding differences in *cytb* expression were 7.69 and 2.88-fold in the virulent and avirulent pathotypes, respectively, 48 hours of 0.25 µg mL^{-1} TDM treatment.

Conclusions: According to findings, it is possible to propose that *cytb* gene might play a role in signaling events during *C. sativus* exposure to commercial triazole fungicide.

Keywords: *Cochliobolus Sativus*, Triazole Resistance, *Cytb* Analysis, qRT-PCR

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Introduction

In recent years, the spot blotch (SB) disease caused by the fungus *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. Ex Dastur (anamorphic: *Bipolaris sorokiniana* (Sacc.) Shoemaker) has emerged as a serious concern for barley grown in warmer and humid regions of the world.[1,2] The use of resistant cultivars is the most practical method of managing SB. However, host resistance is not permanent because cultivar-specific physiological races of *C. sativus* are known to exist and, over the years, have developed on cultivars once thought to be highly SB resistant.[3] Therefore, in the absence of varietal resistance, the most effective SB management practice is to make multiple preventive fungicide applications throughout the growing season.[2]

Fungicides from different chemical groups are approved for use on barley worldwide. The triazole group (e.g. triadimefon; TDM) has been proved to be very effective against SB disease.[4,5] However, it has been reported that *C. sativus* poses a high risk to develop resistance against fungicides like TDM, because of its high genetic variability,[6,7] short life cycle[3] and abundant inoculum production.[8] Therefore, understanding *C. sativus* resistance mechanisms to this common fungicide, can help to establish strategies for sustainable fungicide management in the field.

Triazole fungicides belonging to the sterol demethylation inhibitor group, are characterized by the inhibition of ergosterol biosynthesis, a fundamental component of the fungal cell plasma membrane.[9] The target of these fungicides is lanosterol 14-α demethylase (Erg11 protein), a cytochrome P450 enzyme that is involved in the conversion of lanosterol to 4, 4-dimethylcholesta-8(9), 14, 24-trien-3β-ol. The azole agents are linked to this enzyme using the aromatic five-membered heterocycle and thereby inhibit the cytochrome P450 catalytic activity.[10]

Fungal pathogens can rapidly develop molecular mechanisms of resistance to triazoles as a result of selective pressure by the continued use of regular or sub-regular dosages of fungicide.[5,11] Many molecular studies have reported that the mitochondrial gene for cytochrome *b* (*cytb*) can play a significant role in resistance to fungicides. Sierotzki et al,[12] Kuck and Gisi,[13] and Gisi et al,[14] reported that *cytb* can function in the resistance to quinone outside inhibitor (QoI) fungicides by inhibiting the mitochondrial respiration. Quantitative real-time polymerase chain reaction (qRT-PCR) has been used as a valuable and effective method for measuring changes in mitochondrial gene expressions due to

its high sensitivity.[15,16]

Knowledge of the evolution of fungicide resistance in *C. sativus* population is vital in Syria to evaluate and improve the processing SB disease management program, and for developing management strategies for fungicide resistance. Therefore, the present work aimed to evaluate for the first time the changes in *cytb* gene in two major Syrian *C. sativus* virulent (Pt4) and avirulent (Pt1) pathotypes at early time series of TDM treatment using qRT-PCR.

Materials and Methods

Fungal Isolates

Monoconidial isolates of the virulent (Pt4) and avirulent (Pt1) *C. sativus* pathotypes from Syria[17] were used in this study. The fungus was grown separately in 9 cm Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) with 13 mg/L kanamycin sulphate added after autoclaving and incubated for 10 days, at 22 ±1°C in the dark to allow mycelial growth and sporulation. The cultures were maintained on silica gel at 4°C until needed.

Fungicide

The commercially available fungicide TDM [1-(4-chlorophenoxy)-3,3-dimethyl-1-(1,2,4-triazol-1-yl) butan-2-one] (25% w/v Bayleton, Bayer, India Ltd, Mumbai) was used in this study. It is a member of triazoles that is 1-hydroxy-3, 3-dimethyl-1-(1,2,4-triazol-1-yl)butan-2-one in which the hydroxyl hydrogen is replaced by a 4-chlorophenyl group.

Fungicide Sensitivity

Cochliobolus sativus sensitivity to TDM was determined by measuring the radial growth of each pathotype on PDA plates using the method described by Nene and Thapliyal.[18] To do so, TDM was added to PDA medium after sterilization to give final concentrations of 0.125, 0.25, 0.312 and 0.625 µg/mL TDM. A 10 mm mycelial plug was taken from the edge of a 5-day-old colony and was placed on the center of PDA plates amended with each concentration of the fungicide. The PDA medium without fungicide was served as a control. Five replications were maintained for each treatment/pathotype in a complete randomized design. Plates were incubated at 18-20°C for 3 days in the dark and, subsequently, the diameter of the colonies was measured. Relative growth rate (RGR) was calculated by dividing the growth rate of an isolate in the presence of TDM with that observed in the fungicide absence. The EC50 values were calculated as described by Secor and Rivera.[19] Fungal radial growth was measured on each plate in the TDM dilution series, and compared with growth on non-amended PDA medium to calculate an EC50. The experimental data were analyzed by STAT-ITCF statistical programme (2nd *version*). Differences between means were evaluated for significance by using Newman-Keuls test at 5% probability level.[20]

RNA Isolation and cDNA Synthesis

RNA was isolated from mycelia of each isolate at 24, 48, 72 and 96 hours post fungicide treatments using Nucleotrap mRNA mini kit (Macherey-Nagel, MN, Germany) following the manufacturer's protocol. At the same time, mycelia from non-treated Petri dishes were served as a control. The first-strand complementary DNA (cDNA) was then synthesized from 5 µg of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. Also, cDNA was stored at −20°C.

Quantitative Real-Time PCR

Cytb expression was verified by qPCR according to the protocol described by Vielba-Fernández et al.[21] The sequence information for all RT-PCR primers is given in Table 1. *Cytb* expression was assayed in StepOnePlus™, 96 well using SYBR Green Master kit (Roche). All cDNA samples, standards and controls (no genomic DNA) were assayed for the target gene in triplicate in a single run. The threshold cycle (Ct) value was automatically determined for each reaction by the real time PCR system with default parameters. For accurate estimation of PCR efficiency, the standard curve was performed with a StepOnePlus™ software (v2.3). In our triplicate experimental conditions, a standard curve slope of −3.32 indicated a PCR reaction with 100% efficiency whereas, slopes more negative than −3.32 (ex. −3.9) indicate reactions that are less than 100% efficient.

Data Analysis

Raw data of fluorescence levels and the specificity of the amplicons were checked by qRT-PCR dissociation curve analysis using StepOne™ software (v. 2.3). The fluorescence readings of three replicated samples were averaged, and blank value (from no-DNA control) was subtracted. The final Ct values were the mean of three replicates and the coefficient of variance was calculated to evaluate the variation of Ct values for each gene. Each qRT-PCR reaction set included water as a negative no-template control instead of cDNA. The fold change in putative target gene expression levels was determined using the Ct method,[22] with *EF1α* as a reference (housekeeping control) gene. Standard deviation was calculated from the replicated experimental data. The statistical analysis was conducted through the Tukey's test at the 0.05 level.

Results and Discussion

In this study, the resistance of virulent and avirulent *C. sativus* pathotypes to four concentrations of TDM was investigated using RGR and *cytb* gene expression. Data showed that the ratio of RGR was decreased for both pathotypes by increasing TDM concentration, and the maximum mycelial growth

Table 1. Properties and Nucleotide Sequences of Primers Used in This Study

Gene	Gene Description	Sequence	Amplified Fragment (bp)
EF1α	Elongation factor-1 Alpha	GGCTGATTGTGCTGTGCTTA TGGTGGCATCCATCTTGTTA	153
Cytb	Protein coding	AGCAATGCATTACAACCCTAGC CTATTCATGGTATAGCGCTC	223

inhibition by 50% (EC50) was recorded 48 hours at 0.25 µg/mL TDM treatment (Table 2). Results demonstrated that *C. sativus* could grow under low TDM fungicide doses (0.0625 and 0.0321 µg/mL). This observation suggests that low doses of TDM may actually promote the growth of *C. sativus* which might be taken into account when field applications are contemplated.

To better understand TDM resistance, changes in *cytb* gene expression of *C. sativus* were monitored at early time series following TDM treatment using qRT-PCR. Data showed that *Cytb* gene exhibited a differential expression by $P = 0.05$, and was inversely regulated during different time points post of fungicide treatment. However, at 48 hours, *cytb* gene was significantly expressed by8 fold increases in the virulent pathotype Pt4, as compared with controls (Figure 1). These results suggest that *C. sativus* is having a kind of resistance during TDM fungicide *application*, which could be one of the main causes for the observed low efficacy of triazole. These results are in agreement with those of Somani et al[5] who reported that a strong selection pressure during several years and frequent applications of triazoles for SB control lead to emergence of resistant *C. sativus* populations.[5] Similar scenario of intensive triazole usage leading to the emergence of resistance and reduced fungicide efficacy has been reported in Europe, South America, and Asia for many plant pathogens associated with cereal crops such as *Erysiphe graminis* on barley and wheat[23] and *Mycosphaerella graminicola*[24] and *Parastagonospora nodorum* on wheat.[25]

However, although multiple mechanisms may confer fungal resistance against fungicides, they were most frequently triggered by point mutations in the fungicides' target sites such as *cytb* gene, which encodes the enzymatic targets of demethylation inhibitors and QoIs.[26] The mode of action of Q_oIs is based on blocking mitochondrial respiration and thus energy production in the cells, by binding to the cytochrome bc1 enzyme complex (complex III) at the Q_o site which is partially encoded by the mitochondrial *cytb* gene.[26,27]

Therefore, the changes in *Cytb* gene of *C. sativus* found in this study is perhaps due to natural mutations occurred in mtDNA which is believed to exhibit higher mutation rates compared to nuclear DNA, mainly as a result of less efficient

Table 2. Mean Colony Diameter (Mm) of Fungicide Treatment TDM and Non-treatment of *Cochliobolus sativus* Pt1 and Pt4 Isolates

Time	Isolates	Con.	Treatment With TDM			
			0.25	0.125	0.0625	0.0312
24 hours	*Pt1*	F1.30a	A0.30c	C0.74b	D0.85b	F0.90a
	Pt4	F1.45a	A0.30c	C0.80b	D0.85b	F1.10b
48 hours	*Pt1*	E2.50a	A0.40d	C0.90c	D1.20b	E1.50b
	Pt4	D3.44a	A0.50c	B1.50b	C1.80b	E1.90b
72h	*Pt1*	C11.50a	A0.50d	B1.20c	C1.50c	D2.55b
	Pt4	C12.00a	A0.70d	B1.50c	C1.90c	C3.30b
96h	*Pt1*	B36.20a	A0.50e	B1.70d	B2.90c	B6.80b
	Pt4	A40.10a	A0.50e	A2.30d	A3.90c	A11.2b

Means proceeded by different capital letters (raw) and followed by the different lowercase letter (column) and significantly different at ($P < 0.05$) according to the Newman-Keuls test.

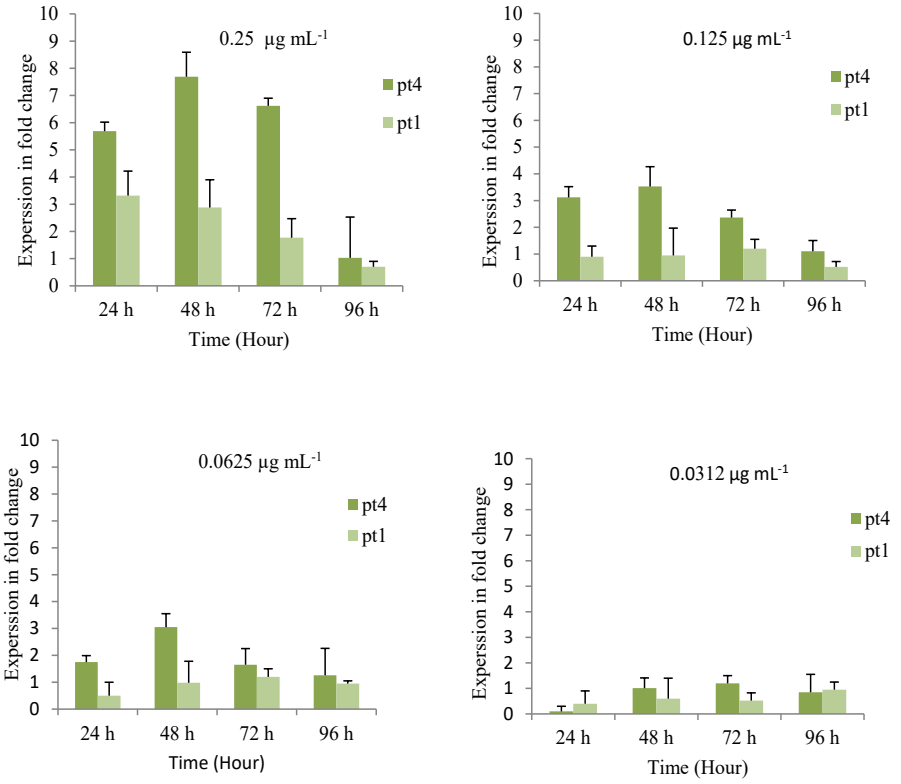

Figure 1. Relative Expression Profiles of *cytb* Gene in the Virulent *Cochliobolus sativus* Pathotype (Pt4) and in a Virulent Pathotype (Pt1) During the Time Course Following Triadimefon Treatments. Error bars are representative of the standard error (Mean ± SD, $n = 3$). Data are normalized to Elongation factor 1α (EF-1α) gene expression level (to the calibrator, Control 0 h, taken as 1.00).

DNA repair mechanisms and a more mutagenic intracellular environment that is created by the production of free radicals during mitochondrial respiration, such as reactive oxygen intermediates.[28,29] However, for deciphering whether these changes in the *C. sativus cytb* gene have been caused by natural mutations further work is required.

In this context, the efficacy of triazole fungicides can be affected due to cross-resistance or when an isolate develops resistance to all fungicides in a chemical group.[30] Different works have also suggested that cross- and multidrug-resistance may be driving forces in the development of resistance in fungi that are at the interfaces of agroecosystem, domestic, and hospital environments.[31,32] However, due to the polygenic nature of *C. sativus* resistance attributed to TDM fungicides, the resistance to this kind of fungicides found against the common pathotype Pt4 within Syrian barley fields may be a result of slow and gradual selective pressure exerted on the pathogen populations due to a long-term use of TDM fungicides at high dosages, as well as due to the potential of migration of the resistance trait through sexual or asexual reproduction.[5] Moreover, it has been shown for certain phytopathogens that resistance mechanisms may develop locally and subsequently spread across countries.[23]

Conclusions

The results of the present study revealed that *cytb* expression gene increased in both virulent and avirulent pathotypes at early time points following TDM application in comparison with non-treated ones, which is of value to give us an indicator about its role in signaling events during exposure to triazole fungicide.

In addition, *C. sativus* had an ability to grow under low TDM fungicide doses, this observation should be taken into account when field applications are contemplated. To avoid this resistance over the next few years, the adoption of anti-resistance management strategy is urgently needed.

However, to decrease the selective pressure towards resistant *C. sativus* populations, rotations of fungicides with different modes of action should be applied[33] along with adoption of mixtures of single-target-site, high-risk fungicides with multiple-target-site and low-risk fungicides.[29]

Authors' Contributions

All experiments, the manuscript writing and its finalization were carried out by MA, HA, and MJ. EA was involved in the statistical analysis of data.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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