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Mechanisms of Resistance to Triadimefon, an Azole Fungicide in the Barley Spot Blotch Fungus, *Cochliobolus sativus*

M. Jawhar*, E. Al-Shehadah, H. Alek

[1]*Department of Molecular Biology and Biotechnology, AECS, P. O. Box 6091 Damascus, Syria*

Abstract

Spot blotch, caused by *Cochliobolus sativus* is an economical disease of barley worldwide. Resistance *C. sativus* to Triadimefon (TDM) a triazole compound fungicide has emerged due to its high genetic diversity, short life cycle and plentiful spore production; however, mechanism(s) underlying such TDM-resistance remains unclear. In the current work, expression of four well known genes involved in fungicide resistance mechanisms *cytb, MFS*, *ABC1* and *ABC2* were monitored in a highly virulent *C. sativus* strain (*Cs*16) at early points of TDM treatments using quantitative real-time PCR (qPCR). Data showed 50% mycelial growth inhibition (EC50) was recorded after 48h at 0.25 µg mL^{-1} TDM treatment. The qRT-PCR revealed significant variance in the expression patterns of the four genes at 24 hours post TDM treatments as compared to the non-inoculated controls. It is also noteworthy that maximum expressions for *cytb* (7.69 -fold), *MFS* (6.11 -fold), *ABC1* (*3.4* -fold) and *ABC2* (1.7-fold) were recorded at different time points of TDM treatments. The obtained results suggest that these genes, positively activated in *C. sativus* during TDM applications, which can provide testable hypotheses that will need direct future tests to decide how these changes may be specified in the genome of the virulent *C. sativus* pathotype.

Keywords: *Cochliobolus sativus*, triazole resistance, gene expression, qRT-PCR

1. Introduction

Cochliobolus sativus (Ito & Kuribayashi) Drechs. Ex Dastur (anamorphic: *Bipolaris sorokiniana* (Sacc.) Shoemaker) is an ascomycetous fungus that causes spot blotch of barley-which is responsible for heavy crop losses in warmer and humid regions worldwide [1, 2]. In Syria, this disease has become greater significance due to the increasing demand for barley as well as the intensive management practices [3]. Recently, *C. sativus* has become more significant because of the rapid change in pathotype patterns and agricultural practices [4].

In the last decades, various fungicides from different chemical groups have been used on barley worldwide. The triazole group (e.g. triadimefon; TDM) has been proved to be very effective against spot blotch disease [5]. However, it has been reported that *C. sativus* poses a high danger to build up resistance against TDM fungicide, due to its high genetic variability and abundant spore production [6, 7].

It is highly challenging to control this pathogen due to a poor understanding of the resistance mechanisms to this common fungicide. Therefore, increasing our understanding of these mechanisms is still needed.

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 which are regulated through various plant signaling pathways [8]. One of these mechanisms involves the overexpression of genes that control the efflux of azoles out of fungal cells. Genes associated with efflux of fungicides are mainly ATP-binding cassette (ABC) transporters or major facilitator superfamily (MFS) transporters [9]. In addition, the mitochondrial gene for cytochrome *b* (*cytb*) can play a significant role in resistance to fungicides by inhibiting the mitochondrial respiration [10]. However, relatively little is known about gene expression of *cytb, MFS, ABC1* and *ABC2* during

Corresponding authors: E-mail: ascientific@aec.org.sy

C. sativus treating with TDM fungicide. Quantitative PCR (qPCR) would be simple and effective method for measuring the changes in relative expression level of genes a result of physiology, pathophysiology or development, because it can detect and precisely quantify very small amounts of specific nucleic acid sequences [11].

Knowledge of the development of TDM fungicide resistance in *C. sativus* population in Syria is critical to increase the effective use of fungicides and decrease the expenses of spot blotch management. Thus, it would be theoretically important to address molecular background of *C. sativus* causing their TDM resistance. The current work aimed to evaluating for the first time the changes in induction of *cytb, MFS*, *ABC1* and *ABC2* expressions in the highly *C. sativus* virulent strain (Cs16) pathotype at early time series of TDM treatment using PCR (qPCR) approach.

2. Materials and methods

Fungal pathotype. The Syrian *C. sativus* strain (*Cs*16) has proved to be the highly virulent pathotype on barley differential sets under field and greenhouse experiments for over 15 years [5,12]. Therefore, it was chosen and used in this study. The fungus was incubated in Petri dishes containing potato dextrose agar (PDA) medium (DIFCO, Detroit, MI. USA) for 10 days at 20–22 °C in the dark.

Fungicide. The commercially available fungicide triadimefon (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) against SB was used in this investigation. It is a systemic triazole fungicide 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.

Sensitivity tests. *C. sativus* sensitivity to TDM was determined by measuring the radial growth on PDA plates as described by Nene and Thapliyal [13]. To do so, TDM was added to PDA medium after sterilization to make the following series of final concentrations 0.0312, 0.0625, 0.125 and 0.25 µg mL^{-1} TDM. A 10 mm mycelial plug was punched out from the margins of a 5-day-old fungal colony and placed on the center of PDA plates amended with each concentration of TDM. The PDA medium without fungicide was used as a control treatment. Each treatment contained six replicates and all experiments were performed at least two times. Plates were incubated at 18 - 20 ºC for 3 days in the dark and, subsequently, the diameter of the colonies was measured. Relative growth rate (RGR) was determined by dividing the growth rate of fungus isolate in the presence of TDM with that detected in the fungicide absence. RGR was assessed on each plate in the TDM dilution series, and compared with growth on non-amended PDA medium to calculate EC50 according to Secor and Rivera [14]. Data were analyzed by STAT-ITCF statistical programme (2nd *Version*), and differences between means were evaluated for significance by using Newman-Keuls test at 5% probability level [15].

RNA isolation. mRNA was extracted from mycelium of *C. sativus* Cs16 at 24, 48, 72 and 96 hours post TDM treatments using Nucleotrap mRNA mini kit (Macherey-Nagel, MN, Germany). At the same time points, mycelia from non-treated Petri dishes were used as a control. First strand complementary DNA (cDNA) was synthesized using the Quanti Tect Reverse Transcription Kit (Qiagen) per the manufacturer 's instructions.

Quantitative real-time PCR (qPCR). The expressions of the four studied genes were assayed according to the method of Livak and Schmittgen [16] using SYBR Green Master kit (Roche). The sequence of RT-PCR primers is given in Table 1. The threshold cycle (Ct) value was automatically determined for each reaction by the real time PCR system with default parameters. The final Ct values represented the mean of three replicates and the coefficient of variance was calculated to evaluate the variation of Ct values. For accurate estimation of PCR efficiency, the standard curve was performed with a StepOnePlusTM software (v2.3).

Data analysis. The relative expression levels were measured using the average cycle threshold (Ct). Average Ct values were calculated from the triplicate experiment conducted for each gene, with the ΔCT value determined by subtracting the average Ct value of genes from the Ct value of the *EF1α* gene. Finally, the equation $2^{-\Delta\Delta CT}$ was used to estimate relative expression levels, and the fold change in putative target gene expression levels was determined as described by Livak and Schmittgen [16], with *EF1α* as a reference (housekeeping control) gene. Standard deviation was calculated from the replicated experimental data.

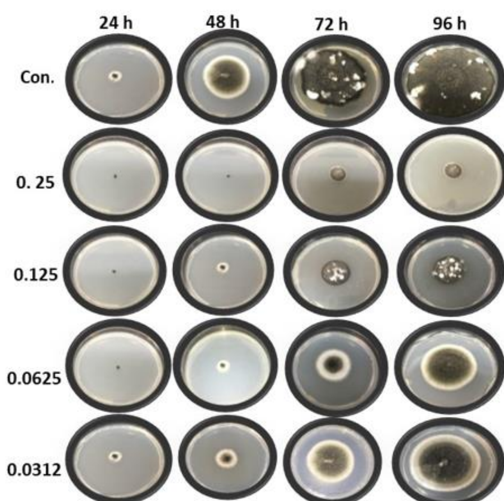
The statistical analysis was performed through the Tukey's test at the 0.05 level.

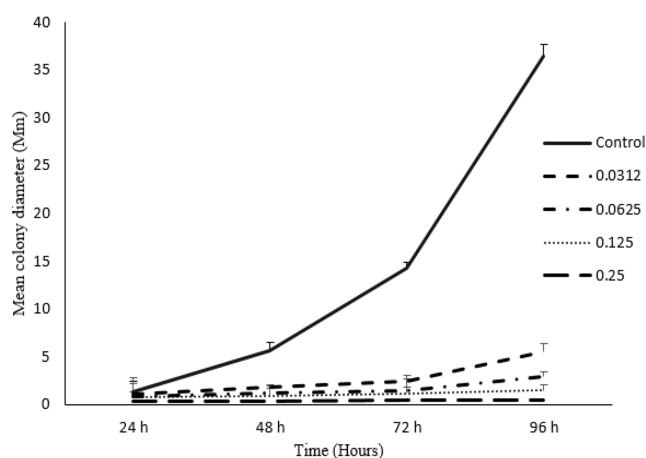
3. Results and discussions

In this work, the resistance of the virulent *C. sativus* strain *Cs*16 to four concentrations of TDM was investigated using RGR and expressions of four well known genes *cytb, MFS*, *ABC1* and *ABC2* involved in fungicide resistance mechanisms. Data showed that the ratio of RGR was decreased of the strain Cs16 by increasing TDM concentration, and the maximum mycelial growth inhibition by 50% (EC50) was recorded 48 hours at 0.25 µg/ mL TDM treatment (Table 1). 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 (Fig.1).

Table 1. Properties and nucleotide sequen of primers used in this study

Gene	Gene description	Sequence
EF1α	Elongation foctor-1 Alapha	GGCTGATTGTGCTGTGCTTA TGGTGGCATCCATCTTGTTA
MFS	Superfamily of membrane transport proteins	TCCACGGTCAGACCAGGCCC CGCGGACTGGTAGGTCGAGGT
CYTB	Protein coding	CCAATGACATGAAAATCATCGTT TCTCCATTTCTGGTTTACAAGAC
ABC1	ATP_ bining cassette transporter	GCCTGGCAGGTGGAAGACAAATAC ATGGCCAAAATCACAAGGGGTTAGC
ABC2	ATP_ bining cassette transporter	TGTGTGGGCAACTGCATCG GTTGGTTTCCATTTCAGATGACATCCG





A B

Figure 1. **(A)** *In vitro* sensitivity of the virulent *C. sativus* strain (*Cs*16) to different concentrations of TDM treatments. **(B)** Mean colony diameter (mm) of fungicide treatment (µg mL^{-1}). Error bars are representative of the standard error (Mean ± SD, *n* = 3).

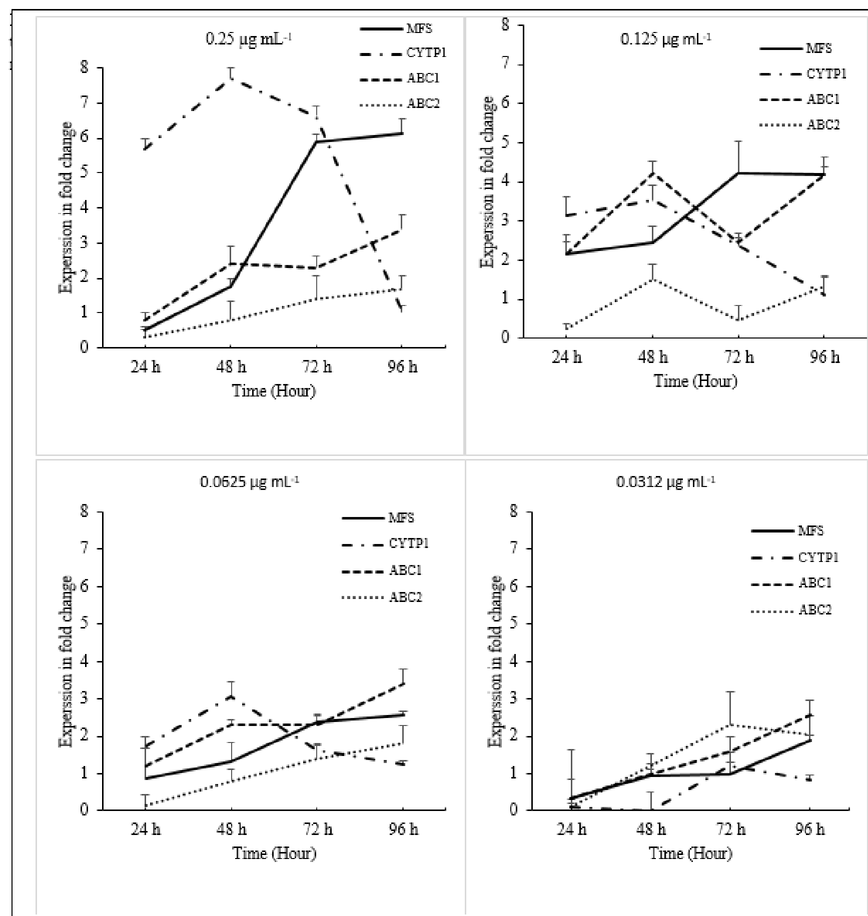


Figure 2. Relative expression profiles of, *MFS*, *cytb, ABC1* and *ABC2* genes in the virulent *C. sativus* strain (*Cs*16) during the time course following triadimefon treatments. Error bars are representative of the standard error (Mean ± SD, *n* = 3). Data are normalized to Elongation factor 1α (EF-1α) gene expression level (to the calibrator, Control 0 h, taken as 1.00).

To better understand TDM resistance, changes in *cytb, MFS*, *ABC1* and *ABC2* gene expression of *C. sativus* virulent strain Cs16 were monitored at early time series following TDM treatment using qRT-PCR. Data showed that *these* genes exhibited a differential expression by $P = 0.05$, and was inversely regulated during different time points post of fungicide treatment. However data showed maximum expression for *cytb* (7.69 -fold), *MFS* (6.11 -fold), *ABC1* (*3.4-* -fold) and *ABC2* (1.7-fold) at different time points of TDM treatments (Fig. 2).

These genes have various signaling pathways against fungicides due to point mutations in the fungicides' target sites such as *cytb* gene, which encodes the enzymatic targets of demethylation inhibitors and QoIs [17]. In addition, it has been also reported that fungal MFS transporters had a functional role in secretion of toxins, and some of them have been shown to protect against fungicides [18,19].

ABCs are also thought to be of very important in adaptation of pathogen to a range of fungicides, since they are capable to bind and hydrolyze nucleotide triphosphates (mainly ATP) due to the possessing of a conserved cytosolic, nucleotide-binding fold (NBF or ATP-binding domain) and utilize this energy to transport solutes through cell membranes [20]. These different mechanisms might propose that the studied genes here might play roles in signaling events during *C. sativus* exposure to commercial triazole fungicide during several growing seasons. These findings can be supported by the results of Somani et al. [5] who reported that a strong selection pressure during several years and frequent applications of triazoles for spot blotch control lead to emergence of resistant *C. sativus* populations.

Similar resistance to triazole was found in *Erysiphe graminis* on barley [21] and in *Mycosphaerella graminicola* on wheat [22].

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 *C. sativus* pathotype [5,12] within Syrian barley fields might be a result of slow and gradual selective stress applied on the pathogen populations due to a long-term use of high doses of TDM fungicides, as well as due to the possible of migration of the resistance trait through sexual or asexual reproduction [5].

The results of the present study revealed significant increases in *cytb, MFS*, *ABC1* and *ABC2* expression were found in the virulent *C. sativus* pathotype at early time points following TDM application as compared with the non-treated ones, which is of value to give us an indicator about their roles in signaling events during exposure to triazole fungicide. It is also noteworthy that *C. sativus* had an ability to grow under very low TDM fungicide doses, this observation should be considered during the field applications. Thus, to avoid this resistance over the next few years, the adoption of anti-resistance management strategy is urgently needed.

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Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors also declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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