# Evaluation of MFS transporter gene expression changes in *Cochliobolus sativus* treated with triadimefon, a triazole fungicide

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## Abstract

*Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur, the causal agent of spot blotch disease, is a necrotrophic pathogen affecting barley production worldwide. Traditional methods to control this disease are commonly used fungicide applications including triadimefon, a triazole compound. *C. sativus* have a great interest since it might expand resistance against triadimefon due to its high genetic variability and abundant spore production. Therefore, understanding of resistance strategies in this fungus is imperative to developing more effective defenses against the spread of resistance. Major Facilitator Superfamily (MFS) transporters play an important role in resistance in fungi. In this study, expression patterns of MFS transporter gene in two *C. sativus* virulent and avirulent pathotypes were monitored at early time periods of triadimefon treatments using quantitative PCR (qPCR) method. Results demonstrated 50% mycelial growth inhibition ($EC_{50}$) for both *C. sativus* pathotypes was recorded after 96h at 0.25 µg $mL^{-1}$ triadimefon treatments. qRT-PCR revealed that MFS expression increased in both pathotypes at 24 h post triadimefon treatments as compared with the corresponding controls. The most outstanding variations in this gene expression were 6.11 and 3.55-fold in the virulent and avirulent pathotypes, respectively, 96h of 0.25 µg $mL^{-1}$ triadimefon treatments. This is the first report on the changes of MFS expression in the fungus *C. sativus*. According to these findings, it is likely to suggest that MFS gene might has a function in signaling events during treatment *C. sativus* with triazole fungicide.

**Keywords:** Barley, *Cochliobolus sativus*, Triazole resistance, MFS analysis, Quantitative PCR

## Introduction

Spot blotch caused by *Cochliobolus sativus* [anamorph: *Bipolaris sorokiniana* (Sacc.) Shoemaker], is one of most important diseases of barley (*Hordeum vulgare* L.) causing huge crop yield losses globally (Kumar *et al.,* 2002). In recent years, spot blotch has also become more significant because of the rapid change in pathotype patterns and agricultural practices (Guo *et al.,* 2019). In Syria, with the increasing demand for barley and more intensive management practices, spot blotch has assumed greater significance (Arabi and Jawhar, 2004).

The most effective and environmentally sound means of control is the use of resistant cultivars, however, barley 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 spot blotch resistant (Gupta *et al.,* 2018). Therefore, in the absence of varietal resistance, the most effective spot blotch management practice is to make multiple preventive fungicide applications throughout the growing season, but their extensive use may lead to the development of resistant pathogen strains (Somani *et al.,* 2019).

In the last decades, numerous cases of fungicide resistance have occurred world-wide, leading to losses of entire fungicide classes in several cases (Leroux *et al.,* 2010; Estep *et al.,* 2015). The emergence of resistance within fungal populations influences most of different fungicide groups (Hawkins, 2015). Triazole fungicides are characterized by the inhibition of ergosterol biosynthesis, an important component of the fungal cell membrane (Snelders *et al.,* 2010). This group (e.g. triadimefon) has been applied as an efficient fungicide to control SB disease, but *C. sativus* has a high danger to develop resistance towards these fungicides, due to its high genetic variability and plenty of inoculum. Therefore, understanding these resistance mechanisms is crucial for sustainable fungicide management in the field.

Many fungal genes have been reported to be involved in fungicide resistance (Pereira *et al.,* 2020), of them, many genes encode putative transporters including major facilitator superfamily (MFS) (Lin *et al.,* 2018). They comprise of wide range transporters to initiate fungicide resistance, including MgMfsl in wheat fungal pathogen *Mycosphaerella graminicola* (Orton *et al.,* 2011), and CaMDRl in *Candida albicans* (Prasad and Kapoor, 2005). These transporter mechanisms confer

fungicide resistance because they export substrates belonging to various chemical groups, which are important to adaptation of fungal pathogen to different fungicides (Deising *et al.,* 2008). Quantitative real-time PCR (qRT-PCR) has been proved as an effective approach for measuring *changes* in transporter gene expressions due to its high sensitivity (Cao *et al.,* 2013).

Genetic design associated with azole resistance appearance remains mostly unknown, therefore, increasing our information regarding the evolution of *C. sativus* resistance can improve our understanding about the processing SB management strategies for fungicide resistance. The present study aimed to evaluate for the first time the changes in MFS transporter gene in major Syrian *C. sativus* pathotypes at early times of triadimefon treatment using qRT-PCR.

# Material and Methods

### *C. sativus* pathotypes

The two major pathotypes of *C. sativus* in Syria, Pt1 (avirulent) and Pt4 (virulent), were used in this investigation (Arabi and Jawhar, 2003, 2004).Mycelia was grown separately in Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) and incubated at 22 ± 1 ºC for 10 days 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

The sensitivity of *C. sativus* to triadimefon was determined by measuring the radial growth of each pathotype on PDA plates using the method described by Nene and Thapliyal (1979). TDM was added to PDA medium after sterilization to give final concentrations of 0.0312, 0.0625, 0.125 and 0.25 µg $mL^{-1}$ TDM. A mycelial plug (10 mm in diameter) 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. PDA medium without fungicide was used as a control. 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. EC50 values were calculated due to Secor and Rivera (2012). RGR was assessed on each plate in the TDM dilution series, and compared with growth on non-amended PDA medium to calculate EC50. Data were analyzed by STAT-ITCF statistical programme ($2^{nd}$ *Version*). Differences between means were evaluated for significance by using Newman-Keuls test at 5% probability level (Anonymous, 1988).

### RNA isolation and cDNA synthesis

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

### Quantitative real-time PCR (qPCR)

MFS expression was assayed in Step One Plus, 96 well using SYBR Green Master kit (Roche). according to the protocol described by Livak and Schmittgen (2001). The sequence information for RT-PCR primers is presented in Table 1. All cDNA samples, standards and controls were assayed in three replications in a single run. The threshold cycle (Ct) was measured by the real time PCR system, and data were verified using StepOne™ Software (v 2.3). The final Ct values represented the mean of three replicates and the coefficient of variance was calculated to evaluate the variation of Ct values. The fold changes in MFS expression levels were measured using the Ct method, with *EF1α* as a reference gene. The statistical analysis was achieved through the Tukey's test at the 0.05 level.

# Results and Discussion

In this work, to better understand triadimefon resistance, changes in MFS transporter gene expression in two virulent and avirulent *C. sativus* pathotypes were monitored at early time periods of triadimefon applications. Data demonstrated that the ratio of RGR was decreased for both pathotypes by increasing triadimefon concentration, and the greatest mycelial growth inhibition by 50% (EC50) was recorded 96 h at 0.25 µg $mL^{-1}$ triadimefon treatment (Fig. 1), and that *C. sativus* fungus could grow under very low triadimefon fungicide rates (0.0321 and 0.0625 µg $mL^{-1}$). This result suggests that triadimefon with low doses might encourage the growth of *C. sativus* which should be considered during the field applications.

On the other hand, results showed that MFS transporter gene exhibited a differential expression by $P = 0.05$, and was inversely regulated during

different time points post of fungicide treatment. However, the most outstanding variations in MFS expression were 6.11 and 3.55-fold in the virulent Pt4 and avirulent Pt1 pathotypes, respectively, 96 h of 0.25 µg mL$^{-1}$ triadimefon treatment (Fig. 2). These data could suggest that *C. sativus* is having a kind of resistance after triadimefon fungicide application, which could be one of the major reasons for the detected low efficacy of triazole. These findings are in line with those of Somani *et al.* (2019) who reported that a high selection pressure during several years and frequent applications of triazoles for SB control lead to emergence of resistant *C. sativus* populations. Similar results of intensive triazole application 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 *Pyricularia* on wheat (Dorigan *et al.,* 2019), and *Erysiphe graminis* on barley and wheat (Buchenauer and Hellwald, 1985).

It has been reported that fungal MFS transporters had a functional role in secretion of toxins (Stergiopoulos *et al.,* 2002), and some of them have been shown to protect against fungicides, such as a MSF gene from *B. cinerea*, showed increasing in sensitivity towards the natural toxic compounds camptothecin and cercosporin, generated by the plant fungal pathogen *Cercospora kikuchii* (Hayashi *et al.,* 2002). However, the resistance *C. sativus* to triadimefon found in this study may be a result of slow and gradual selective pressure exerted on the pathogen populations due to a long-term use of fungicides at high dosages (Walters *et al.,* 2012).

## Conclusion

This study demonstrated for the first time that MFS expression gene increased in both *C. sativus* virulent and avirulent pathotypes at early time periods after triadimefon applications in comparison with controls, which is an indicator about its role in signaling events during exposure to triazole fungicide. In addition, *C. sativus* had an ability to grow under very low triadimefon fungicide doses, this observation should be taken into consideration when field applications are contemplated. However, to avoid this resistance over the next few years, the adoption of anti-resistance management strategy is urgently needed.


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**Table 1:** Sequences of primers used in this work.

| Sequence | Gene description | Gene |
|---|---|---|
| GGCTGATTGTGCTGTGCTTA<br>TGGTGGCATCCATCTTGTTA | Elongation factor-1 Alpha | *EF1α* |
| TCCACGGTCAGACCAGGCCC<br>CGCGGACTGGTAGGTCGAGGT | Superfamily of membrane *transport* proteins | MFS |

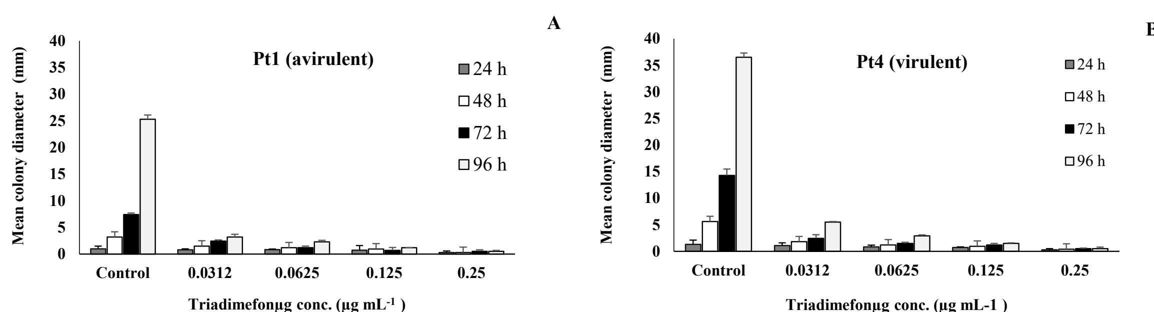


**Fig. 1:** Mean colony diameter (mm) of fungicide treatment (µg mL$^{-1}$) TDM and non-treatment of *C. sativus* Pt1 and Pt4 pathotypes. Error bars are representative of the standard error.

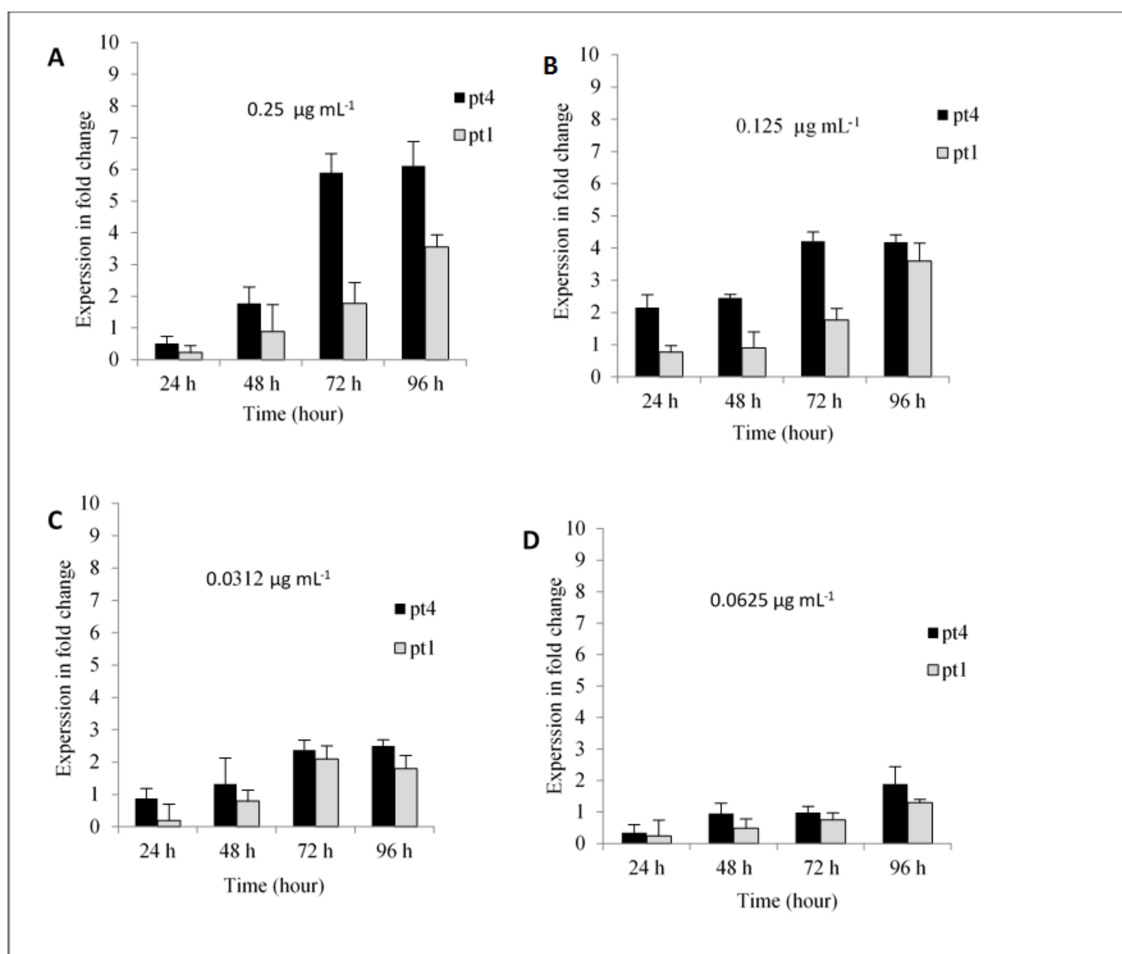


**Fig. 2:** Relative expression profiles of *MFS* gene in *C. sativus* virulent pathotype (Pt4) and a virulent pathotype (Pt1) during the time course following triadimefon treatments. Error bars are representative of the standard error (Mean ± SD, $n = 3$). Data are normalized to Elongation factor 1α (EF-1α) gene expression level (to the calibrator, Control 0 h, taken as 1.00).

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