JAP&T
TPA-Timişoara

Available online at http://journal-of-agroalimentary.ro

Journal of Agroalimentary Processes and Technologies 2021, *27*(3), 291-295

Journal of Agroalimentary Processes and Technologies

Alterations in ABC1 Gene Expression in Two Virulent and Avirulent *Cochliobolus sativus* Pathotypes Treated with Triazole Fungicide

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

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

Abstract

Cochliobolus sativus is the causal agent of barley spot blotch (SB), a disease responsible for serious crop losses. Triadimefon (TDM) a triazole compound is used for controlling SB worldwide. The resistance of *C. sativus* to TDM fungicide has been well documented, but its mechanisms are still poorly understood. ATP-binding cassette (ABC) transporters are believed to be crucial in the adaptation of fungal pathogens to a range of fungicides. In this work, TDM sensitivity of two virulent and avirulent *C. sativus* pathotypes was measured by the relative growth rate at four concentrations and the effective concentration for 50% inhibition (EC_{50}) was determined. ABC1 expression was verified using quantitative reverse transcriptase (qRT-PCR). Results demonstrated that 50% mycelial growth inhibition (EC50) was achieved for both *C. sativus* pathotypes following TDM treatment after 96h at 0.25 µg mL^{-1}. Moreover, qRT-PCR showed intensification of ABC1 transporter in both pathotypes 24h post TDM treatments as compared to controls. Interestingly, the ABC1 expression was greater in the virulent pathotype Pt4 as compared to the avirulent one, Pt1. Together, changes in ABC1 levels after TDM short-term treatment in both *C. sativus* pathotypes suggested its importance in the triazole resistance.

Keywords: Barley, *Cochliobolus sativus*, triazole resistance, ABC1 analysis, qRT-PCR

1. Introduction

The barley spot blotch (SB) fungus, *Cochliobolus sativus* (Ito & Kurib.) Drechsl. ex Dast. (anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.), is an economically important disease worldwide. SB has become more significant due to the rapid changes in pathotype patterns and agricultural practices [8]. Improvement of resistant cultivars is the most suitable way for managing this disease, however, in the absence of varietal resistance [9], the most effective SB management practice is to make multiple preventive fungicide applications during the growing season, but their extensive use may lead to the development of resistant fungal isolates [18].

Triazole (e.g. triadimefon; TDM) has been used as an effective fungicide for controlling SB disease, since, as compared to other systemic fungicides; the specific site of action of triazole is an inherent benefit that has led to improve control effectiveness against the pathogenic fungi [5].

Recently, it has been reported that these fungicides are prone to resistance within fungal pathogen populations, especially without optional practices that are aimed at prolonging the effectiveness of those fungicides [12, 15]. However, *C. sativus* has a high hazard to develop resistance towards these fungicides, which is due to its high genetic variability and inoculum abundance [10].

Among all the resistance mechanisms described, the ATP-binding cassette (ABC) transporters which have a wide specificity spectrum for the elimination of toxic products such as fungicides [20], therefore, many of them have significant roles in fungicide sensitivity and resistance [7, 17]. Schoonbeek et al. [17] reported that ABC transporter BcatrB from *Botrytis cinerea* mediates resistance to the phytoalexin resveratrol and the fungicide fenpiclonil. Resistance to azole fungicides was accompanied with an increase in ABC1 expression in wheat *Mycosphaerella graminicola* [22]. On the other hand, quantitative real-time PCR (qRT-PCR)

Corresponding authors: ascientific30@aec.org.sy

has been proved to be an effective method for measuring *changes* in transporter genes [4].

Information on the evolution of TDM resistance in *C. sativus* is vital to evaluate and improve the currently deployed SB disease management program. Therefore, the purpose of the current work was to evaluate for the first time the alterations in ABC1 gene in two virulent and avirulent *C. sativus* pathotypes at early time points of TDM treatment using qRT-PCR.

2. Materials and Methods

Fungal pathotypes. The two major pathotypes of *C. sativus* in Syria, Pt1 (avirulent) and Pt4 (virulent), were used in this investigation [2, 3]. They were grown separately in 9 cm diameter sterile Petri dishes containing potato dextrose agar (PDA) medium (DIFCO, Detroit, MI. USA) and incubated at 22 ±1ºC 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 work. 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 evaluated by measuring the relative growth rate (RGR) on PDA plates as described by *Nene and Thapliyal* [14]. TDM was added to PDA medium after sterilization to make final concentrations of 0.0312, 0.0625, 0.125 and 0.25 µg mL^{-1} TDM. A mycelial plug (10 mm) 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 the fungicide was used as a control. Each treatment contained six replicates and all experiments were performed two times. Plates were incubated for 3 days at 20 ºC in the dark, and the colonies diameter was measured. RGR was determined by dividing the growth rate of each pathotype in the presence of TDM with that detected in the fungicide absence. EC50 values were calculated due to *Secor and Rivera* [16]. RGR was assessed on each plate in the TDM dilution series, and compared with controls to calculate EC50. Results were analyzed by STAT-ITCF statistical programme [1].

RNA isolation and cDNA synthesis. mRNA was extracted from mycelium of the two pathotypes at 24, 48, 72 and 96 hours post fungicide treatments using Nucleotrap mRNA mini kit (Macherey-Nagel, MN, Germany). Non-treated Petri dishes were served as controls. The first-strand complementary DNA (cDNA) was synthesized by Quanti Tect Reverse Transcription Kit (Qiagen) and stored at −20 °C.

Quantification of ABC expression. The expression of ABC1 was quantified by qRT-PCR using a protocol described by *Livak and Schmittgen* [11]. Primer sequences are presented in Table 1. The threshold cycle (Ct) was measured by the real time PCR system, and data were verified using StepOne™ Software (v2.3). The final Ct values represented the mean of three replicates and the fold change in ABC1 levels was evaluated using the Ct method. *EF1α* gene was used as a reference. Statistical analysis was achieved by Tukey's test at the 0.05 level.

3. Results and discussions

In this work, changes in ABC1 transporter gene expression in two virulent and avirulent *C. sativus* pathotypes were evaluated at early time periods following TDM treatments. Data showed that the mycelial growth inhibition rates increased by 50% (EC50) at 96h as the fungi were exposed to a greater TDM concentration (0.25 µg mL^{-1}) in both pathotypes (Fig. 1), and that mycelia could grow under very low level of TDM fungicide (0.0321and 0.0625 µg mL^{-1}), which should be considered when field applications are desired.

To better understand *C. sativus* resistance to TDM, alterations in ABC1 gene expression were monitored at early time series of TDM treatments using qRT-PCR. Results demonstrated that ABC1 displayed a differential expression patterns which was contrary regulated during TDM treatments. Interestingly, the ABC expression was greater in the virulent pathotype Pt4 as compared with the avirulent one Pt1 at 96h under different TDM treatments (Fig. 2), this might could be one of the major reasons for the detected low efficacy of triazole towards TDM. These findings are in line with those of Somani et al. [18] who reported that frequent applications of triazoles for SB control lead to the emergence of resistant *C. sativus* populations.

A resistance mechanism based on increased ABC transporters expression is regularly outcome in an energy-dependent decreased cellular content of toxicants, since ABC transporters 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 [6, 21]. Therefore, ABCs are thought to be of very important in adaptation of pathogen to a range of fungicides [22]. As a matter of fact, the ABC transporter gene ABC1 of the rice blast fungus *Magnaporthe grisea* [19] is strongly induced by the rice phytoalexin sakuranetin and by azole fungicides. Similarly, ABC1 transporter and disturbance of the encoding gene affected azole-resistant in *Penicillium digitatum* [13].

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
ABC 1	ATP-binding cassette transporter	GCCTGGCAGGTGGAAGACAAATAC ATGGCCAAAATCACAAGGGGTTAGC	145

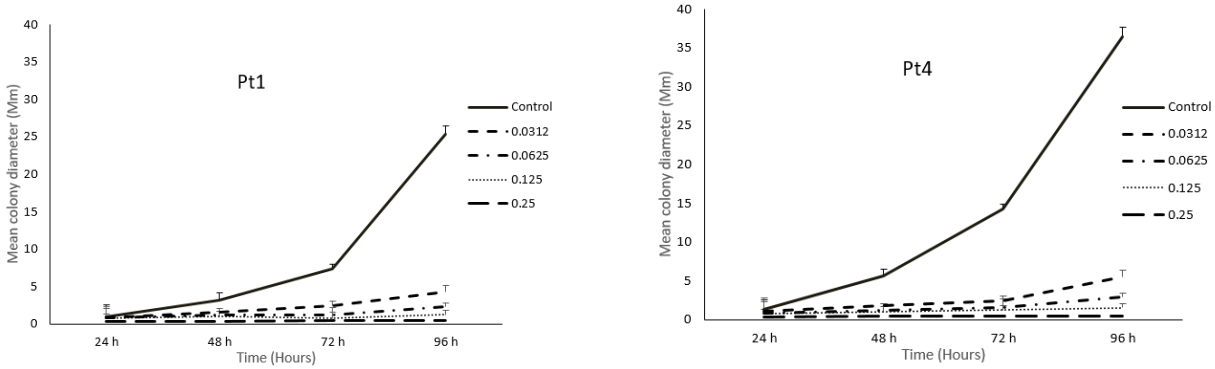

Figure 1. Mean colony diameter (mm) of fungicide treatment (µg mL^{-1}) TDM and non-treatment of *C. sativus* Pt1 and Pt4 isolates. Error bars are representative of the standard error (Mean ± SD, *n* = 3).

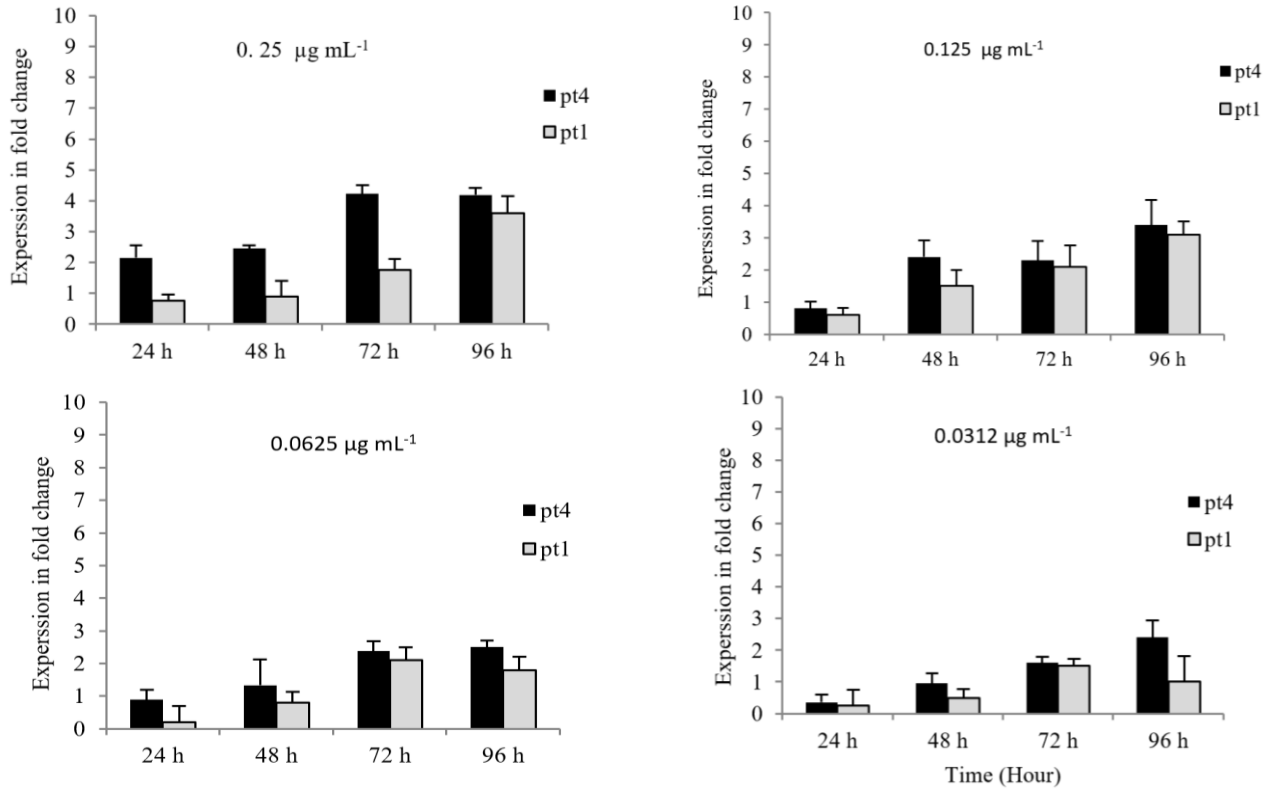

Figure 2. Relative expression profiles of ABC1 gene in the virulent (Pt4) and a virulent (Pt1) *C. sativus* pathotypes during time course following triadimefon treatments. Error bars are representative of the standard error (Mean ± SD, *n* = 3). Data are normalized to Elongation factor 1α (EF-1α) gene expression level (to the calibrator, Control 0 h, taken as 1.00).

The preliminary experiment of qRT-PCR analysis showed that expression levels of ABC1 were higher in the treated samples (Fig. 2), suggesting that ABC1 potentially has an efflux activity against azole fungicides (such TDM). Nevertheless, because overexpression of ABC1 transporter genes conferred azole TDM resistance on both *C. sativus* pathotypes, it would be useful to examine, by a biochemical analysis, whether the ABC1 transporter gene definitely has an ability to export the azole fungicides out of the cell. Furthermore, since a number of ABC transporter genes are being in the fungal genome [6, 17], it is likely that there are some other ABC transporters that potentially have azole efflux activity.

Finally, we presume that the ABC1 transporter might play a role in TDM fungicide resistance, since its expression was increased in both virulent and avirulent *C. sativus* pathotypes at early times of TDM applications as compared with the controls. Interestingly, the ABC1 expression was greater in the virulent pathotype Pt4 as compared with the avirulent one Pt1 under different TDM treatments, which needs further studies. Additionally, *C. sativus* had an ability to grow under very low levels of TDM fungicide which should be considered during the field applications. However, the resistance of *C. sativus* to TDM found in this investigation may be the result of slow and gradual selective pressure exerted on the pathogen populations due to a long-term use of fungicides at high dosages [20]. Therefore, to pass up this resistance in future, the adoption of anti-resistance management plan is urgently required.

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

Acknowledgements: The authors would like to thank the Director General of AECS and the Head of Molecular biology and Biotechnology Department for their much appreciated help during the period of this work. Thanks are also extended to Dr. A. Al-Daoude for c reading of the manuscript.

References

1. Anonymous. 'STAT-ITCF, Programme, MICROSTA, realized by ECOSOFT, 2nd Ver.' (Institut Technique des Cereals et des Fourrages: Paris), **1988,** pp.55.
2. Arabi MIE, Jawhar M. Pathotypes of *Cochliobolus sativus* (spot blotch) on barley in Syria. *J. Plant Pathol.* **2003**, *85,* 193-196.
3. Arabi MIE, Jawhar M. Identification of *Cochliobolus sativus* (spot blotch) isolates expressing differential virulence on barley genotypes in Syria. *J. Phytopathol.* **2004**, *152*, 461-464.
4. Cao H, Cao F, Roussel AM, Anderson RA. Quantitative PCR for glucose transporter and tristetraprolin family gene expression in cultured mouse adipocytes and macrophages. *In Vitro Cell Dev. Biol. Anim*. **2013,** *49*,759-770.
5. Hawkins N. The Evolution of fungicide resistance. *Adv. Appl. Microb.* **2015,** *90,* 92.
6. Higgins CF. ABC transporters: from microorganisms to man. *Annu. Rev. Cell Biol.* **1992,** *8,* 67–113.
7. Gomez AO, Mattner SW, Oag D, Nimmo P, Milinkovic M, Villalta ON. Protecting fungicide chemistry used in Australian strawberry production for more sustainable control of powdery mildew and leaf blotch. *Acta Hortic.* **2016,** *1156,* 735–42.
8. Guo H, Yao Q, Chen L, Wang F, Lang X, Pang Y, Feng J, Zhou J, Lin R, Xu S. Virulence and molecular diversity in the *Cochliobolus sativus* population causing barley spot blotch in China. *Plant Dis.* **2019,** 103, 2252-2262.
9. Gupta PK, Vasistha NK, Aggarwal R, Joshi AK. Biology of *B. sorokiniana* (syn. *Cochliobolus sativus*) in genomics era. *J. Plant Biochem. Biotechnol.* **2018,** *27*, 123–138.
10. Kumar J, Schafer P, Huckelhoven R, Langen G, Baltruschat H, Stein E, Nagarajan S, Kogel HK. *Bipolaris sorokiniana*, a cereal pathogen of global concern: cytological and molecular approaches towards better control. *Mol. Plant Pathol.* **2002,** *3*,185-195.
11. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, **2001,** *25*, 402-408.
12. Massi F, Torriani SFF, Borghi L, Toffolatti SL. Fungicide resistance evolution and detection in plant pathogens: *Plasmopara viticola* as a case study. *Microorganisms*. **2021,** *9*, 119.
13. Nakaune R, Adachi K, Nawata O, Tomiyama M, Akutsu K, Hibi T. A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. *Appl. Environ. Microbiol.* **1998,** *64*, 3983–3988.
14. Nene YL, Thapliyal PN. Fungicides in plant disease control. *Oxford and IHB publ. Co., New Delhi.* **1979.**
15. Ribas AD R, Spolti P, Del Ponte EM, Donato KZ, Schrekker H, Fuentefria AM. Is the emergence of fungal resistance to medical triazoles related to their use in the agroecosystems? A mini review, *Braz. J. Microbiol.* **2016,** *47*, 793-799.

16. Secor GA, Rivera VV. Fungicide resistance assays for fungal plant pathogens. in: Plant fungal pathogens: Methods and protocols. *Bolton MD, Thomma BPHJ. eds. Humana Press, New York.* **2012,** 385-392.
17. Schoonbeek H, Del Sorbo G, De Waard MA. The ABC transporter BcatrB affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. *MPMI.* **2001,** *14*, 562–571.
18. Somani D, Adhav R, Prashant R, Kadoo NY. Transcriptomics analysis of propiconazole-treated *Cochliobolus sativus* reveals new putative azole targets in the plant pathogen. *Funct. Integr. Genom.* **2019,** *19*, 453-465.
19. Urban M, Bhargava T, Hamer JE. An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. *EMBO J.* **1999,** *18*, 512-521.
20. Walters DR, Avrova A, Bingham IJ, et al. Control of foliar diseases in barley: towards an integrated approach. *Eur. J. Plant Pathol.* **2012,** *133*, 33–73.
21. Ye Z, Lu Y, Wu T. The impact of ATP-binding cassette transporters on metabolic diseases. *Nutr. Metab. (Lond)* **2020, *17,*** 61.
22. LH, Stergiopoulos L, Van Nistelrooy JGM, De Waard MA. ABC Transporters and azole susceptibility in laboratory strains of the wheat pathogen *Mycosphaerella graminicola*. *Antimicrob. Agents Chemother.* **2003,** *46*, 3900-6.