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Impact of *Bacillus thuringiensis* on inhibiting certain *Alternaria alternata*'s mycotoxins isolated from infected potatoes

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ABSTRACT

Aims: Potatoes are considered one of the most strategic vegetable crops all over the world. *Alternaria alternata* has recently contaminated certain potatoes farms in different regions in Egypt. Among thirteen samples from fifteen regions were studied in a precedent study. Our study was aimed to investigate the effect of *Bacillus thuringiensis* subsp. *Kurosaki* suspension on inhibiting the growth of the three tested isolates of *A. alternata* and minimizing their mycotoxins production *in vitro* using three isolates with three levels of highly, moderate and low pathogenicity with unequal amounts of dual mycotoxins production.
Methodology and results: Three isolates of *A. alternata* from three regions, Kom Hamada (KH3), Alamin (Alam1) and Nobaria (NO3), which were determined as a producer of tenuazonic acid (TeA) and alternariol monomethyl ether (AME) toxins. *Bacillus thuringiensis* (Bt) use as commercial fungicide was applied with three suspension concentrations (75, 150 and 300 µg/mL) as inhibitor for the two mycotoxins. Our results illustrated that the three tested isolates recorded high TeA and AME inhibition efficacies by increasing the Bt suspension concentration. The highest inhibitory concentration of Bt was at concentration 75 µg/mL for isolated from Nobaria third region (NO3) and Alam1 it was (99.83 and 99.74%) for mycotoxin (AME) while, TeA mycotoxin had the most inhibition percentage (99.58%) at concentration 150 µg/mL for the isolate (NO3).
Conclusion, significance and impact of study: The preliminary results of the study suggest that *B. thuringiensis* spores' suspension with different concentrations can be used as anti-mycotoxigenic agents to inhibit the (TeA) and (AME) mycotoxins produced by *Alternaria alternata*.

Keywords: Alternaria alternata, *Bacillus thuringiensis*, TeA, AME inhibition

INTRODUCTION

The necrotrophic fungus *Alternaria alternata* (Fr.) Keissler is an ever-present fungus that contains different pathotypes that produce different mycotoxins and cause numerous plant diseases and extreme damage to crops in the field (Pinto and Patriarca, 2017; Shi *et al.*, 2021). This disease has appeared in several countries and plant crops (Vučković *et al.*, 2013; Pinto and Patriarca, 2017; Heflish *et al.*, 2020). In addition to causing economic losses in production and processing (Kosiak *et al.*, 2004). AAL toxins released by *Alternaria alternata* can reduce grain quality (Vučković *et al.*, 2013; Heflish *et al.*, 2020) and cause different toxicological effects (Youssef *et al.*, 2021). *Alternaria* sp. mycotoxins are well known to have estrogenic activity always lower than zearalenone (ZEN) estrogenic activity, the alternariol momethyl ether (AME) has the most potent estrogenic activity (Dellafiora *et al.*, 2017). Moreover, the presence of these compounds in the food chain is an increasing concern for human and animal health due to their possible harmful effects (Loi *et al.*, 2020). There is a wide range of *Alternaria* spp. toxins, but only a few of them are associated with health risk acute toxicity (Arcella *et al.*, 2016). Alternariol monomethyl ether (AME) is one of the major concerns since researchers has detected a mutagenic, genotoxic and carcinogenic effects of it on mammalian cells (Heflish *et al.*, 2020; Youssef *et al.*, 2021).

Tenuazonic acid (TeA) is non-host selective phytotoxin produced by *A. alternata*, which causes necrosis in many plants (Zhou *et al.*, 2019; Shi *et al.*, 2021; Youssef *et al.*, 2021). Also, the cytotoxic effects on cultured mammalian cells had decrease of the total protein concentrations and inhibition of the proliferation of these mammalian normal cell lines (i.e., 3T3 mouse fibroblasts, Chinese hamster lung cells and human hepatocytes) (Shi *et al.*, 2019). *Bacillus thuringiensis* (Bt) spores were used as insecticides in ancient Egypt

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(Sanahuja *et al.*, 2011) and the isolation and study of this bacterium have attracted many microbiologists. In 1901, the bacterium was isolated and identified in Japan by Shigetane Ishiwatari and named it *Bacillus sotto*. After ten years, the same bacterium was re-isolated in the German province of Thuringia by Ernst Beliner and its name was changed to *Bacillus thuringiensis* (Siegel, 2000; Sanahuja *et al.*, 2011). *Bacillus thuringiensis* is a soil-borne bacterium that produces Cry and or Cyt proteins that are also considered as endotoxins to certain herbivorous insects. The toxin produced by *B. thuringiensis* has been used as an insecticide spray since the 1920s and is commonly used in organic farming. It is also the source of the genes used to genetically modify food crops so that they produce the toxin on their own to deter various insect pests. The toxin is lethal to several orders of insects, including Lepidoptera (butterflies, moths and skippers) (Sanahuja *et al.*, 2011), but is very safe for female mammals (Rubio-Infante and Moreno-Fierros, 2016) found no mortality with *B. thuringiensis*, indicating a high level of safety when compared to *B. anthracis* (the most virulent *Bacillus* to mammals). For example, the LD_{50} values of *B. anthracis*-derived spores were low, from 2.64 to 80 for intraperitoneal or subcutaneous injection compared with dosages used to assess the safety of *B. thuringiensis* products one million- to one trillion-fold higher (10^{12} spores for mice). *Bacillus thuringiensis* is not toxic to humans or other mammals but is harmful to certain insects when ingested (Betz *et al.*, 2000). The Environmental Protection Agency (EPA) and numerous scientific bodies have consistently found that *B. thuringiensis* and engineered *B. thuringiensis*-crops are not harmful to humans (Koch *et al.*, 2015). In the last decade, potato plants producing Cry 3A *B. thuringiensis* toxin were approved safe by the Environmental Protection Agency.

Our study was aimed to investigate the effect of the *B. thuringiensis* on inhibiting the mycotoxins produced by three different isolates of *Alternaria alternata* in order to limit the use of chemical pesticides and using a safe insecticide to investigate its efficacy in reducing these mycotoxins in order to protect the environment and save human health.

MATERIALS AND METHODS

Fungal isolates origin

Three isolates of *Alternaria alternata* with high mycotoxins production capability were obtained from a previous study (Youssef *et al.*, 2021) which isolated from three different regions Kom Hamada (KH3), Alamin (Alam1) and Nobaria (NO3). These isolates have different pathogenic degrees and different phylogenic types. They were considered as producers of tenuazonic acid (TeA) and alternariol monomethyl ether (AME) (Youssef *et al.*, 2021).

Preparation of the fungal culture

Each fungal strain was inoculated onto potato dextrose agar (PDA) in Petri dishes and incubated under alternative fluorescent white light for 7 days at 25 °C in an incubator (WTC binder type BIS N° = 87602/Germany), after which the colony surface was gently scraped off and transferred to a tube containing 50 mL of sterile distilled water. The spores were counted using a hemocytometer, each one mL containing approximately 1×10^4 spores/mL (Youssef *et al.*, 2021).

Bacillus thuringiensis origin

Bacillus thuringiensis subsp. *Kurosaki*, a mesophilic lyophilized powder produced by Valent Biosciences Corporation (United States) which produces exoenzymes was brought from May Trade Company. ARE was provided by Prof. Abdel Fatah Saad from Department of Plant Protection, Alexandria University, Egypt. According to Rocha's research, this Bt strain has proteolytic and amylolytic properties (Rocha *et al.*, 2016). 75 µg/mL, 150 µg/mL and 300 µg/mL were the concentrations used in this study. These concentrations are less than the recommended applied dose as insecticides which contain around 2000 bacterial cell/mL because the higher amount of detoxifier agent the lower detoxification efficacy (Youssef, 2012).

Preparation of the *B. thuringiensis* stock solution

The broth culture of *B. thuringiensis* spores was obtained by activating bacterial strain inoculum in Brain Heart Broth (Difco) for 24 h at 25 °C according to Youssef and Sabra (2021) with certain modifications. The spores were harvested individually by centrifugation at 600 rpm for 10 min and then washed twice with a phosphate buffer and turbidity was adjusted to an optical density (O.D.) of 0.85 at 600 nm using a spectrophotometer. The cell suspension (200 µL) was re-suspended into Erlenmeyer flasks containing 250 mL of Nutrient Broth (Difco) and incubated at 30 °C for 72 h. One litre of the working solution of *B. thuringiensis* spores' suspension solution was prepared for each concentration 75, 150 and 300 µg/mL of *B. thuringiensis* spores suspension solution successively.

Preparation of the experiment *in vitro*

Three groups contained 12 Erlenmeyer flasks (250 mL) each. Replicates are repeated thrice. Each flask was filled with 100 mL of Potato Dextrose Broth (PDB) then inoculated with each tested *Alternaria alternata* isolate of each selected region. Three replicates were used also for non-treated control where each group of flasks (12 flaks) was autoclaved then inoculated with the desired *Alternaria* inoculum 5 mL of spores suspension containing around (1×10^4 spores/mL) (Youssef *et al.*, 2021) then incubated for seven days at 25 °C. At the end of the incubation period, the tested concentration of *B.*

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Table 1: Effect of *Bacillus thuringiensis* concentrations on TeA produced by *A. alternata* isolate (1) infected potato crop in Khom Hamada region (KH3).

Bacillus thuringiensis concentrations (µg per mL)	Mycotoxin concentrations ng/g (TeA)	TeA efficacy inhibition ratio (%)
Control (0)	$121213^{a} \pm 103.21$	
75	$4882^{b} \pm 6.74$	95.97
150	$898.6^{c} \pm 8.11$	99.26
300	$886^{d} \pm 7.97$	99.27
$LSD_{0.05}$	1.633	

*The data are means of three replicates ± the standard deviation. Data with the same letter do not differ significantly and the data with the different letters differ significantly at $p<0.05$.

Table 2: Effect of *B. thuringiensis* concentrations on AME produced by *A. alternata* isolate (1) infected potato crop in Khom Hamada region (KH3).

Bacillus thuringiensis concentrations (µg per mL)	Mycotoxin concentrations ng/g (AME)	AME efficacy inhibition ratio (%)
Control (0)	$117988^{a} \pm 73.05$	
75	$454^{c} \pm 7.11$	99.62
150	$612.4^{b} \pm 8.03$	99.48
300	$353.7^{d} \pm 3.18$	99.70
$LSD_{0.05}$	1.338	

*The data are means of three replicates ± the standard deviation. Data with the same letter do not differ significantly and the data with the different letters differ significantly at $p<0.05$.

thuringiensis was added at their three concentrations. All flasks were incubated for fifteen days at 27 °C and then the flasks were taken for mycotoxins detection.

Mycotoxin detection process

Each tested mycotoxin was detected by taking an aliquot 20 mL each was taken and each mycotoxin. TeA and/or AME was estimated by using HPLC analysis in liquid culture according to Siciliano *et al.* (2015) and Youssef *et al.* (2021) and the resulting data were registered.

Statistics analysis

The experiment was performed in triplicates in a completely randomized design. The generated data was statistically analyzed by using one-way ANOVA, and the results were compared by the least significant difference $p \leq 0.05$ according to Duncan's Multiple Range test using Costat statistical package (CoHort Software, CA, USA) (Duncan, 1955; McDonald, 2014). At the same time, the statistical differences in the mean were determined by the standard deviation (± SD).

RESULTS AND DISCUSSION

Estimation of TeA and AME in treated and non-treated *Alternaria alternata* media

The effect of *B. thuringiensis* on the TeA and AME toxins for the three *Alternaria alternate* isolates were investigated in Tables (1-6). The data exhibited that *B. thuringiensis* spores' suspension highly inhibited both mycotoxins produced by the three tested *Alternaria* isolates. Furthermore, the same mycotoxin produced by different fungi does not have the same sensitivity. This finding was also matched with (Alshannaq *et al.*, 2018), who reported that twenty-six *Aspergillus flavus* isolates differed in their aflatoxin production capabilities. Results illustrated that the *A. alternata* isolate of Khom Hamada (KH3) produced TeA more than AME, which indicated that the same fungus could produce more than one mycotoxin in unequal amounts. The findings are consistent with those reported by Youssef (2019), who revealed that *Penicillium verrucosum* produces two mycotoxins, ochratoxin and citrinin, in unequal amounts, however the same mycotoxin produced by different fungi has different sensitivity. This finding was also matched with (Heflish *et al.*, 2020) who reported that citrinin produced by *Penicillium citrinum* does not have the same particle properties of the citrinin produced by *P. verrucosum*. Our results also exhibited that the isolate of Alam1 was the most tolerant to *B. thuringiensis* spores' suspension for both tested mycotoxins followed by KH3 isolate in case of TeA inhibition only. This last-mentioned finding indicated that the same fungal species might vary in its cell wall properties and consequently its degree of pathogenicity, so its susceptibility to biological control agent is not the same (Youssef *et al.*, 2021).

Comparison between *Bacillus thuringiensis* concentration efficacies against *A. alternata* isolates mycotoxins inhibition

Each tested mycotoxin varied in its production according to the produced *Alternaria alternate* isolate behavior and

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Table 3: Effect of *B. thuringiensis* concentrations on TeA produced by *A. alternata* isolate (2) infected potato crop in Alamin region (Alam1).

Bacillus thuringiensis concentrations (µg per mL)	Mycotoxin concentrations ng/g (TeA)	TeA efficacy inhibition ratio (%)
Control (0)	$37223^{a} \pm 76.41$	
75	$1910.5^{b} \pm 12.52$	94.87
150	$1154.5^{c} \pm 7.21$	96.90
300	$889.5^{d} \pm 9.15$	97.61
$LSD_{0.05}$	0.9464	

*The data are means of three replicates ± the standard deviation. Data with the same letter do not differ significantly and the data with the different letters differ significantly at $p<0.05$.

Table 4: Effect of *B. thuringiensis* concentrations on AME produced by *A. alternata* isolate (2) infected potato crop in Alamin region (Alam1).

Bacillus thuringiensis concentrations (µg per mL)	Mycotoxin concentrations ng/g (AME)	AME efficacy inhibition ratio (%)
Control (0)	$147277^{a} \pm 147.2$	
75	$385.96^{c} \pm 4.21$	99.74
150	$679.22^{b} \pm 7.73$	99.60
300	$357.32^{d} \pm 3.94$	99.76
$LSD_{0.05}$	0.9415	

*The data are means of three replicates ± the standard deviation. Data with the same letter do not differ significantly and the data with the different letters differ significantly at $p<0.05$.

Table 5: Effect of *B thuringiensis* concentrations on TeA produced by *A. alternata* isolate (3) infected potato crop in Nobaria third region (NO3).

Bacillus thuringiensis concentrations (µg/mL)	Mycotoxin concentrations ng/g (TeA)	TeA efficacy inhibition ratio (%)
Control (0)	$49201^{a} \pm 85.94$	
75	$282.6^{c} \pm 6.23$	99.43
150	$206.6^{d} \pm 2.10$	99.58
300	$733.8^{b} \pm 7.33$	98.51
$LSD_{0.05}$	0.28792	

*The data are means of three replicates ± the standard deviation. Data with the same letter do not differ significantly and the data with the different letters differ significantly at $p<0.05$.

Table 6: Effect of *B. thuringiensis* concentrations on AME produced by *A. alternata* isolate (3) infected potato crop in Nobaria third region (NO_3).

Bacillus thuringiensis concentrations (µg per mL)	Mycotoxin concentrations ng/g (AME)	AME efficacy inhibition ratio (%)
Control (0)	$209268^{a} \pm 901.84$	
75	$360.25^{d} \pm 9.22$	99.83
150	$701.5^{b} \pm 7.10$	99.67
300	$695.63^{c} \pm 3.84$	99.67
$LSD_{0.05}$	0.9461	

*The data are means of three replicates ± the standard deviation. Data with the same letter do not differ significantly and the data with the different letters differ significantly at $p<0.05$.

the *B. thuringiensis* spores' suspension concentration as follows:

In the case of TeA: Figure 1 showed that the isolate of NO3 (Nobaria third region) was the most sensitive fungus to *B. thuringiensis* suspension at concentration 75 µg/mL followed by Kom Hamada third region isolate (KH3) then Alamin first region isolate (Alam 1). Both the tested fungal isolates of KH3 and Alam1 realized the highest TeA inhibition efficacy by using *B. thuringiensis* suspension and at concentration 300 µg/mL whereas, the isolate of NO3 realized its best TeA inhibition at concentration 150 µg/mL, which indicated that this isolate (NO3) was less resistant than the other two tested fungi (Tables 1, 3 and 6).

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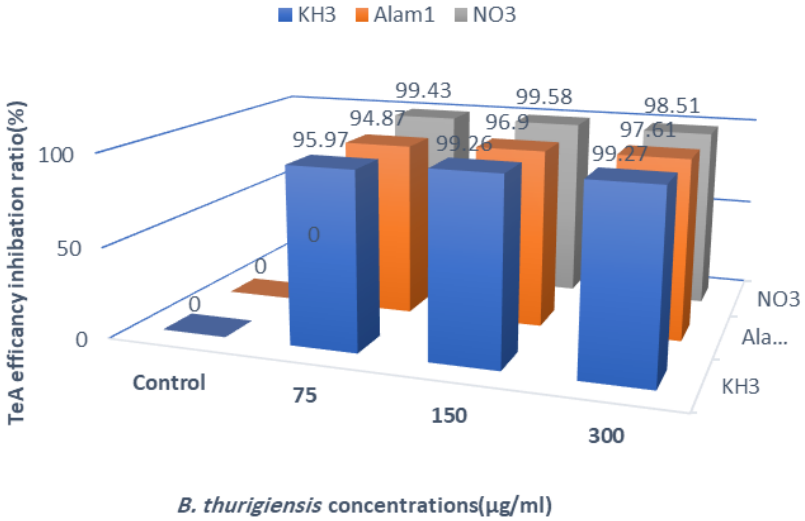

Figure 1: Comparisons between the efficacy percentages of *B. thuringiensis* concentration suspension in reducing the TeA mycotoxin produced by *A. alternata* isolates. KH3, Khom Hamada region; Alam1, Alamin region; NO3, Nobaria third region. Each control efficacy % of the three tested isolates = 0.

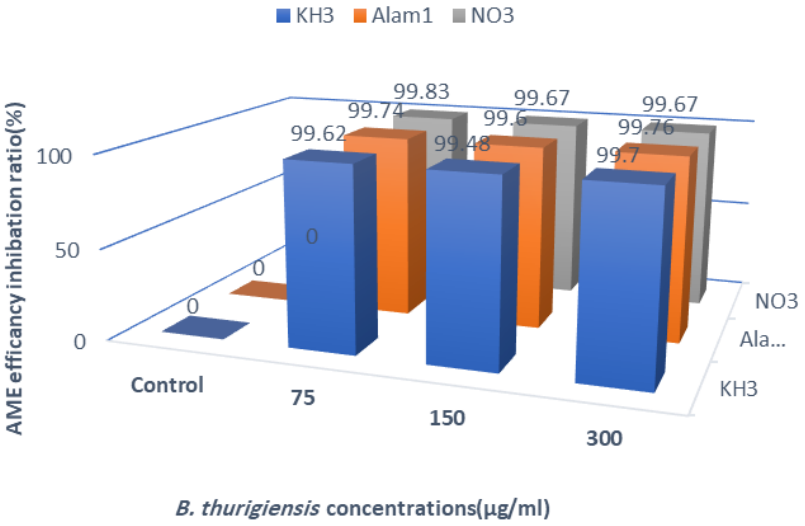

Figure 2: Comparisons between the efficacy percentages of *B. thuringiensis* concentration suspension in reducing the AME mycotoxin produced by *A. alternata* isolates. KH3, Khom Hamada region; Alam1, Alamin region; NO3, Nobaria third region. Each control efficacy % of the three tested isolates = 0.

In the case of AME: At both concentrations of 75 and 150 µg/mL, the NO3 isolate was the most sensitive, followed by Alam1 isolate then KH3 isolate whereas at concentration 300 µg/mL, the isolate KH3 was the most suppressive isolate by *B. thuringiensis* suspension followed by Alam1 then NO3 isolates (Figure 2). We noticed that the behaviour of the same fungal isolate under the same biotic treatment differed in their dual mycotoxins production. Our findings are in match with Youssef (2019), who reported that *P. verrucosum*, which produced dual mycotoxins citrinin and ochratroxin each mycotoxins varied in its behaviour under the same inhibition process our same results was shown in Tables 2, 4 and 5.

Data, as exhibited, illustrated that both mycotoxins of the isolate of KH3 and Alam1 were less inhibited by *B. thuringiensis* spores suspension than the same dual mycotoxins of the isolate of NO3, which indicated that not all the same mycotoxins have the same susceptibility and the fungal strain of the produced mycotoxin is a very important factor in the inhibition process. Moreover, our results are closely in the harmony of Youssef (2019) and Daou *et al.* (2021) who mentioned that the fungus strain was among factors that affect the mycotoxin's

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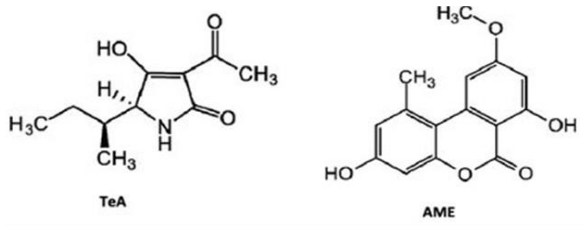

Figure 3: Structure of both tested mycotoxins in this study (Stroka and Gonçalves, 2019).

contamination process. Our results revealed that the effectiveness of *B. thuringiensis* spores' suspension in reducing TeA and AME are not similar that's may be due to the difference between the properties and the chemical structure of the two tested mycotoxin (Figure 3). Our findings are in agreement with Rocha *et al.* (2016), who reported that *B. thuringiensis* was effective in reducing fumonisin B1 but not effective at reducing fumonisin B2, which produced by the same fungus *Fusarium verticilloides*.

On the other hand, our data can be matched with Pazzi *et al.* (2006), Abbas *et al.* (2013) and Rocha *et al.* (2016) who reported that *B. thuringiensis* was effective in reducing fumonisin B1 but not effective in reducing aflatoxins thrichocethins and zearalenone. The findings of Rocha *et al.* (2016) demonstrated that *B. thuringiensis* had a strong influence on *F. verticillioides in vitro* while also inhibiting fumonisin production. Using the flow cytometry analysis illustrated the fungal cell oscillations and death during these interactions which determine the efficacy of *B. thuringiensis* subsp. *Kurstaki* in controlling plant pathogens.

Mode of action of *B. thuringiensis* spores' suspension

Bacillus thuringiensis's mode of action against TeA and AME can be explained by the action of the outer membrane protein A from bacteria *Pantoea* sp. which can bind aflatoxin particles (Xie *et al.*, 2019). Multiple fungal metabolites such as ergot alkaloids are degraded by ErgA from bacteria *Rhodococcus erythropolis* (Hahn *et al.*, 2015) and mycotoxins can be degraded by bacteria (Taheur *et al.*, 2019). The combination of *B. thuringiensis* protein such as Cry1Ab with other Cry proteins (such as Cry1F) or Vip proteins has reduced the incidence of pests and indirectlymycotoxin levels (Díaz-Gómez *et al.*, 2016).

CONCLUSION

Bacillus thuringiensis spores' suspension has a strong inhibitory activity against TeA and AME produced by *A. alternaria* isolates.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest. The authors certify that they have no link with any organization with any interest in the research. All co-authors have seen and agreed with the contents of the manuscript and there is no financial interest to report.

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