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SHORT COMMUNICATION

The fig-tree skeletonizer moth, *Choreutis nemorana* (Hübner, 1799) (Lepidoptera: Choreutidae), a new species for the Algeria fauna

H. Belguerri[1], B.A. Boulaouad[1]*, S. Bella[2]*, M. Belkacem[3], B. Harzallah[4] and B. Bakhouche[5]

Summary The fig-tree skeletonizer moth, *Choreutis nemorana* (Hübner, 1799) (Lepidoptera, Choreutidae), is a widespread species in the Mediterranean region. In October 2023 and June 2024, during pest control operations as part of the harvesting campaign in the fig groves of the Bordj Ghedir and Wilaya de Bouira regions (Algeria), *C. nemorana* was documented for the first time in Algeria.

Additional keywords: Africa, Algeria, *Choreutis nemorana,* fig-tree skeletonizer moth, first record

Introduction

The fig tree (*Ficus carica* L.) is a highly prevalent plant species in the Mediterranean region. It represents one of the oldest cultivated fruit trees globally and possibly the earliest domesticated plant of the Neolithic revolution, being cultivated approximately a thousand years before cereals (Kislev *et al*., 2006). In Algeria, fig trees have been cultivated for centuries, renowned for their exceptional quality (Abdelkader *et al*., 2023). Alongside olive and citrus, fig trees stand out as economically and socially significant fruit species, comprising over 10% of the national arboricultural heritage. According to FAO (2023), the cultivated area for fig trees in Algeria was 39.065 ha in 2021. A notable decline from the 80.000 ha was recorded in 1950 (Rebour, 1952). In 2021, the fig tree production in the country reached 107.266 tons (FAO, 2023) with a relatively low yield, not surpassing 2.75 tons per ha. These statistics highlight that fig tree cultivation in Algeria persists in a traditional manner, being part of an extensive farming system.

There are few studies on the pests affecting the fig tree in Algeria; the species studied include: *Hypoborus ficus* Erichson and *Hypocryphalus scabricollis* (Eichhoff) (Curculionidae, Scolytinae), *Niphona picticornis* (Mulsant) and *Trichoferus fasciculatus* (Faldermann) (Cerambycidae), *Scobicia chevrieri* (A. Villa and G.B. Villa) and *Sinoxylon sexdentatum* (Olivier) (Bostrichidae), *Lagria viridipennis* (Tenebrionidae, Lagriinae), *Ceroplastes rusci* (Hemiptera, Coccidae), *Paratylenchus* sp. (Nematoda, Tylenchidae) (Biche *et al*., 2012; Chelli *et al*., 2023; Mellal *et al*., 2023).

In this account we report the first occurrence in Algeria of the fig-tree skeletonizer moth, *C. nemorana* (Hübner, 1799) (Lepidoptera: Choreutidae), a monophagous species on *F. carica* (Moraceae).

[1] Laboratory of Characterization and Valorisation of Natural Resources (LCVRN), Mohamed El Bachir El Ibrahimi de Bordj Bou Arréridj,34030 Algeria.
[2] (CREA) Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria - Centro di Ricerca Olivicoltura, Frutticoltura e Agrumicoltura. Corso Savoia, 190, 95024 Acireale (CT), Italy.
[3] Laboratoire Biotechnologies et Protection des Ecosystèmes Agricoles et Naturels, Faculté des Sciences de la Nature et Sciences de la Terre, Université de Bouira, 1000 Bouira, Algeria.
[4] Algerian Wildlife Watchers Association, Algeria.
[5] Laboratory of Biological Oceanography and the Marine Environment (LOBEM), Faculty of Biological Sciences, University of Science and Technology of Houari Boumediene (USTHB), BP 32, El Alia, Bab Ezzouar, 16111 Algiers, Algeria.
* Corresponding authors:
belkacemaimene.boulaouad@univ-bba.dz
salvatore.bella@crea.gov.it

Choreutis nemorana (Hübner, 1799)
Tortrix nemorana Hübner [1799]: pl. 1, fig. 3.

Examined material

Algeria - Bordj Ghedir region, Bordj Bou Arreridj (35°53′ N, 4°53′ E), 1 ♀, 05-X-2023; Wilaya de Bouira region, Bouira 1 ♂, 12-VI-2024, leg. et coll. B.A. Boulaouad.

Results and discussion

The first specimen of *C. nemorana* (Fig. 1) was observed on 5 October, 2023, during the fig harvest period in the Bou Arreridj territory, located to the south of the provincial capital and on 12 June, 2024, in the Bouira territory, in Algeria. In the first site the moth was observed flying from a fig tree to a nearby lemon verbena (*Aloysia citrodora* Paláu) (Verbenaceae). The specimen was identified based on the specific wavy wing shape: forewings mainly reddish brown to ochreous brown, suffused with black and marked extensively with white to grey scales; hindwings brownish, each with a pair of pale spots towards the margin (Alford, 2007). The damage caused by this species on fig trees includes significant skeletonization of leaves, resulting in visible deformation, discoloration, and tearing of the laminae (Alford, 2013).

Choreutis nemorana is a widely distributed species in the Mediterranean region, including Southern Europe and North Africa (Fig. 2). It is also present in the Canary Islands and Madeira, and in parts of Asia (Armenia, Georgia, Iran, Iraq, Azerbaijan, and Uzbekistan) (Rota *et al*., 2014; Lepiforum, 2024). In Europe, it has been observed in Albania, Austria, Belgium, Bosnia and Herzegovina, British Isles, Bulgaria, Croatia, Czech Republic, Cyprus, France (including Corsica), Germany, Gibraltar, Greece (including Crete, Aegean and Dodecanese Islands), Hungary, Italy (including Sicily and Sardinia), Macedonia, Malta, Portugal, Romania, Serbia, Slovakia, Spain (including Balearic Islands), Switzerland, the Netherlands, Turkey, and Ukraine (Gaedike, 2008; De Prins and De Prins, 2014; De Prins *et al*., 2014; Werno, 2014; Szabóky, 2015; Vossen, 2015; Fauster, 2016; Lendel, 2017; Vaneva-Gancheva, 2017; Stojanović *et al*., 2020; Singh *et al*., 2022; Šumpich *et al*., 2023; Lepiforum, 2024). *Choreutis nemorana* was observed in North Africa in Tunisia (Zouba, 2010) and Egypt (El-Abbassi *et al*., 1997) but no prior observations have been made in Algeria and Morocco. Thus, the current record represents the first documented observation of this species in Algeria.

Figure 1. Fig-tree moth *Choreutis nemorana* (Hübner) on *Aloysia citrodora* in Bordj Ghedir region, Algeria.

Regarding the introduction of *C. nemorana* in Algeria, global plant trade and climate change offer great opportunities for numerous insect species to occupy new territories and most likely, these aspects are also having an impact on the spreading of *C. nemorana* in the Mediterranean basin. In fact, the moth has rapidly expanded its distribution area northwards and eastwards during the last 20 years (Vaneva-Gancheva, 2017; Stojanović *et al*., 2020; Lepiforum, 2024).

The cultivation of fig trees in Algeria is conducted within a traditional extensive system that heavily relies on natural resources and the intrinsic characteristics of cultivated varieties. The improvement of current situation in terms of surface area, yield, and production depends on various factors, with a primary emphasis on cultural practices at the orchard level. In addition to the modernization of the irrigation systems and the establishment of effective fertilization programs, controlling pests and diseases in this cultivation has become an absolute necessity for

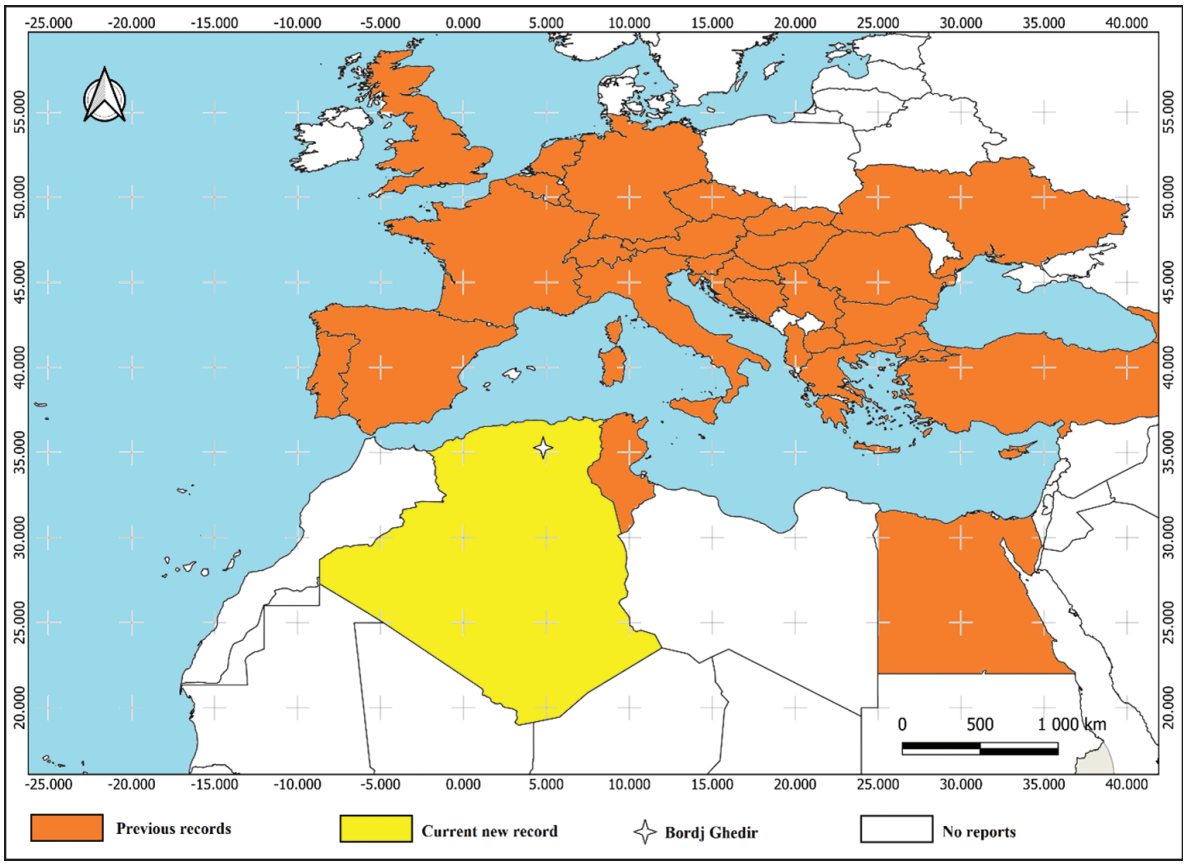

Figure 2. Distribution of *Choreutis nemorana* (Hübner) in Northern Africa - Yellow indicates the new record, orange represents the countries where the species was recorded before, and white indicates no reports.

the preservation of this traditional species (Di Silvestro *et al*., 2021). Notably, *C. nemorana* poses a new threat to fig growers, necessitating national awareness campaigns for the pest recognition (harmful stages, number of generations, etc.). *Choreutis nemorana* is considered a minor pest of *F. carica*, that mainly attacks abandoned, spontaneous, and neglected plants. Control measures are performed by removing and destroying the infested leaves with the caterpillars. In these agroecological conditions, it is important to encourage the spread of the natural enemies of the fig-tree skeletonizer moth, i.e. tachinid diptera, and numerous generic predatory insects (Baviera *et al*., 2017; Singh *et al*., 2022). Furthermore, the development of a control strategy is crucial, emphasizing prevention as the primary approach and considering insecticide treatment as a last resort. Chitgar *et al.* (2014) examined digestive enzyme inhibitors in the gut of *C. nemorana* in the perspective of application in transgenic plants as a safe method against the pest while minimizing environmental impact.

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Choreutis nemorana (Hübner, 1799) (Lepidoptera: Choreutidae), ένα νέο είδος για την πανίδα της Αλγερίας

H. Belguerri, B.A. Boulaouad, S. Bella, M. Belkacem, B. Harzallah και B. Bakhouche

Περίληψη Ο εντομολογικός εχθρός της συκιάς, *Choreutis nemorana* (Hübner, 1799) (Lepidoptera, Choreutidae), είναι ένα ευρέως διαδεδομένο είδος στην περιοχή της Μεσογείου. Τον Οκτώβριο του 2023 και τον Ιούνιο του 2024, κατά τη διάρκεια εφαρμογών φυτοπροστασίας στο πλαίσιο της συγκομιδής σε οπωρώνες συκιάς των περιοχών Bordj Ghedir και Wilaya de Bouira (Αλγερία), τεκμηριώθηκε η παρουσία του *C. nemorana* για πρώτη φορά στην Αλγερία.

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Comparative effect of biopesticides against the fall armyworm *Spodoptera frugiperda* (J.E. Smith)

A.A.M. Atta[1], A.A. Al-Ayat[1], H.A. Gad[1]* and S.A.M. Abdelgaleil[2]

Summary The current study evaluated the effect of microbial-derived insecticides (abamectin and spinosad), two microbials (*Beauveria bassiana* (Balsamo) Vuillemin, *Bacillus thuringiensis* Berliner), and three chitin synthesis inhibitors (CSIs) (chlorfluazuron, hexaflumuron and lufenuron) against *Spodoptera frugiperda* larvae. Spinosad and abamectin caused pronounced mortality against second larval instar of *S. frugiperda* using the leaf dipping method. Spinosad induced higher toxicity (LC_{50} = 4.01 mg/L) than abamectin (LC_{50} = 8.33 mg/L) one day after treatment. The treatments with *B. bassiana* and *B. thuringiensis* caused higher mortality of *S. frugiperda* larvae 7 days after treatment with LC_{50} values of 3.0 × 10^5 spores/ml and 8.2 × 10^6 cells/ml, respectively. In the case of the CSIs, hexaflumuron showed higher toxicity than chlorfluazuron and lufenuron with LC_{50} values of 0.01, 0.009 and 0.005 mg/L 3, 7 and 10 days after treatment, respectively.

Additional keywords: abamectin, *Beauveria bassiana*, *Bacillus thuringiensis*, chitin synthesis inhibitors, fall armyworm

Introduction

Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae) is a destructive insect species for many crops including field crops, such as maize, rice, sorghum, sugarcane, and cotton, and vegetable crops, such as tomato, potato, cucumber, and cabbage. The insect causes massive loss crop yield either in quality or quantity (Murúa *et al.,* 2006; Prasanna *et al.,* 2018). Recently, the insect has become a key pest in grain crops, especially maize, in most of the countries in America and Africa (Rwomushana *et al.,* 2018; Mendesil *et al.,* 2023). Typical insect damage symptoms include holes in plant leaves and death of a heart of young plants due to larvae feeding (Abrahams *et al.,* 2017; Capinera, 2017).

In Egypt, *S. frugiperda* was first recorded in May 2019 in a maize field in Aswan Governorate (Upper Egypt) and since then it has been spread out throughout the country (Dahi *et al.,* 2020; Gamil, 2020; Al-Ayat *et al.,* 2022). *Spodoptera frugiperda* was first reported in West Africa in late 2016 (Goergen *et al.*, 2016), and by early 2017 the pest invaded Sub-Saharan Africa. Recent reports confirmed the occurrence of fall armyworm in 28 countries in Africa (Day *et al.*, 2017). Since the outbreak of *S. frugiperda* in Africa, the synthetic insecticides have been broadly applied for the management of this insect pest on infested crops, particularly maize (Tepa-Yotto *et al.*, 2022). As the overuse of synthetic insecticides is connected with serious problems, such as the increase of environmental pollution, adverse effects on animals and humans, and emerging resistance of insects (Yu, 1991; Prasanna *et al.,* 2018), alternative strategies, such as bioinsecticides, entomopathogenic fungi, pheromone traps, and parasitoids, have been examined and used against *S. frugiperda* (Mendez *et al.,* 2002; Gutierrez-Martinez *et al.,* 2012; Varshney *et al.,* 2021).

Many studies report the efficacy of biopesticides, such as entomopathogenic bacteria, fungi, viruses and microbial-derived insecticides (spinosad, spinetoram, and abamectin) on larvae of *S. frugiperda* (Polanc-

[1] Plant Protection Department, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt.
[2] Department of Pesticide Chemistry and Technology, Faculty of Agriculture, 21545-El-Shatby, Alexandria University, Alexandria, Egypt.
* Corresponding author: hassangad1985@azhar.edu.eg

zyk *et al.,* 2000; Mendez *et al.,* 2002; Molina-Ochoa *et al.,* 2003; Ríos-Velasco *et al.,* 2010; Deshmukh *et al.,* 2020; Kulye *et al.,* 2021; Han *et al.,* 2023). However, no information is available on the efficacy of such products against the Egyptian strain of *S. frugiperda*. Therefore, the study focuses on examining the effect of microbial-derived insecticides (abamectin and spinosad), the microbials (*Beauveria bassiana* (Balsamo) Vuillemin) and *Bacillus thuringiensis* Berliner and three CSIs (chlorfluazuron, hexaflumuron and lufenuron) on *S. frugiperda* larvae in Egypt. Also, the latent effects of CSIs on biological aspects of this insect pest were assessed.

Materials and Methods

Source and Insect Rearing

Larvae of *S. frugiperda* were first collected from a maize field in Al-Sharqia, Egypt and reared on maize leaves until completing a life cycle (Sayed *et al.,* 2022). Healthy male and female adults were allowed to mate and female laid eggs in plastic jars. The resulting neonate larvae were fed on fresh castor bean leaves, *Ricinus communis*, under laboratory (28±1°C, 65±5% relative humidity (RH) and 12:12 h of light and dark). The larvae were reared on castor bean leaves because the plant is available all the year and is cultivated in an area free from insecticides.

Tested insecticide compounds

The tested insecticides were: spinosad (98%) and hexaflumuron (95%) (Dow AgroSciences LLC, USA); abamectin (98%) and lufenuron (94%) (Syngenta, Switzerland); chlorfluazuron (95%) (Simonis BV, Netherlands). The microbial compounds were produced as follows:

Culture of *Beauveria bassiana*

The original source of *B. bassiana* fungus was Bioinsecticides Production Unit, Agriculture Research Center, Giza, Egypt. The fungal strain was cultured and maintained following a protocol described by Mohamed *et al.* (2018). The fungal spores were prepared and the concentration was calculated using a haemocytometer and adjusted to 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7, 1.0×10^8 and 1.0×10^9 spores/ml to be used in the bioassay experiments.

Bacillus thuringiensis

Bacillus thuringiensis subsp. kurstaki was used as a Protecto product (9.4% WP contain 32,000 international unit/mg) produced by Bioinsecticides Production Unit, Agriculture Research Center, Giza, Egypt. The six concentrations of *B. thuringiensis* (3.0×10^5, 3.0×10^6, 3.0×10^7, 3.0×10^8, 3.0×10^9 and 3.0×10^{10} cells/ml).

Bioassays

Bioassays were conducted by using the leaf dipping method according to Insecticide Resistance Action Committee (IRAC) method (IRAC, 2018). The concentrations of tested insecticides, spinosad, abamectin, chlorfluazuron, hexaflumuron and lufenuron were prepared in acetone and tested at 0.01, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 mg/L. Acetone was used as solvent because the active ingredients of insecticides are not soluble in water. The concentrations of *B. bassiana* and *B. thuringiensis* were prepared in distilled water. The fungus (*B. bassiana*) was tested at 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7, 1.0×10^8 and 1.0×10^9 conidia/ml, while *B. thuringiensis* was tested at 32.0×10^4, 32.0×10^5, 32.0×10^6, 32.0×10^7, 32.0×10^8 and 32.0×10^9 cells/ml.

The castor bean leaves were cut into small pieces (4 × 4 cm). The pieces were immersed for five seconds in each concentration and then left to complete evaporation of solvent. Three treated pieces were transferred to each plastic cup (8 cm diameter × 5 cm high). Ten newly molted second instar larvae of *S. frugiperda* were introduced to each cup. The second larval instar was chosen to give sufficient time to complete the experiment before the larvae turned into pupae. The cups were covered with cheese cloth and kept under above mentioned insect rearing environment. Three replicates were used in each tested concentration. An additional series of castor bean leaves were

treated with pure acetone (99%) or distilled water alone and served as a control. After 24 h, the treated leaf pieces were discarded and fresh untreated leaf pieces were introduced daily for 10 days.

Mortality percentages were recorded 1, 3, 5, 7 and 10 days after treatment. Additionally, the larvae fed on the treated leaves with CSIs were examined daily until complete pupation and adult emergence and percentages of pupation, adult emergence and malformation were calculated.

Data Analysis

Abbott's formula (1925) was used for correction of mortality data. Values of LC_{50} were calculated using probit analysis (Finney, 1971). Percentages of pupation, adult emergence and malformation were analyzed with ANOVA using Tukey's HSD test at a significance level <0.05 (SPSS, Chicago, IL, USA).

Results

The mortality of larvae in control treatments, both water and acetone did not exceed 10 and 15%, respectively; the mean of these mortalities was used to correct the larvae mortalities by Abbot for the insecticide treatments, and for the estimation of corresponding LC_{50} values.

Toxicity of the two microbial-derived insecticides (abamectin and spinosad) expressed as LC_{50} values against the second larval instar of *S. frugiperda* 1 and 3 days after treatment is summarized in Table 1. Both insecticides showed pronounced toxicity in leaf dipping application; spinosad showed higher toxicity (LC_{50} = 4.01 mg/L) than abamectin (LC_{50} = 8.33 mg/L) one day after treatment. However, both compounds showed similar toxicity 3 days after treatment where LC_{50} values were 0.18 and 0.19 mg/L for spinosad and abamectin, respectively.

The LC_{50} values of the entomopathogenic fungus *B. bassiana* and the bacterium *B. thuringiensis* against *S. frugiperda* second instar larvae 3, 5 and 7 days after treatment are presented in Table 2. Both biological control agents displayed different levels of insecticidal effect which enhanced with the increase of concentration and time after treatment. The toxicity of *Beauveria bassiana* and *B. thuringiensis* was high 3 days after treatment as their LC_{50} values were 3.9 $x10^7$ spores/ml and 2.6 $x10^8$ cells/ml, respectively. The effect of both biological control agents increased significantly 5 and 7 days after treatment. Five days after treatment, the LC_{50} values for *B. bassiana* and *B. thuringiensis* were $1.2x10^6$ spores/ml and $2.2x10^7$ cells/ml, whereas the values decreased to $3.0x10^5$ spores/ml and $8.2x10^6$ cells/ml, after

Table 1. Comparative toxicity of microbial-derived insecticides against second instar larvae of *Spodoptera frugiperda* 1 and 3 days after treatment.

Insecticide	Time (days)	LC_{50}[a] (mg/L) (Confidence limits)	LC_{90}[b] (mg/L) (Confidence limits)	Slope[c] ± SE	(χ^2)[d]	*P*[e]
Abamectin	1	8.33 (4.49-21.65)	684.71 (163.36-7321.31)	0.67±0.08	4.11	0.391
	3	0.19 (0.12-0.30)	4.47 (2.74-10.96)	0.94±0.07	13.9	0.031
Spinosad	1	4.01 (2.67-7.32)	151.90 (51.32-986.94)	0.81±0.11	5.40	0.144
	3	0.18 (0.13-0.23)	3.53 (2.37-5.98)	0.99±0.09	5.03	0.284

[a,b]The concentration causing 50 and 90% mortality.
[c] Slope of the concentration-mortality regression line ± standard error.
[d] Chi square value.
[e] Probability value.

Table 2. Comparative toxicity of *Beauveria bassiana* and *Bacillus thuringiensis* against second instar larvae of *Spodoptera frugiperda* 3, 5 and 7 days after treatment.

Insecticide	Time (days)	LC_{50}[a] (spores/ml) (Confidence limits)	LC_{90}[b] (spores/ml) (Confidence limits)	Slope[c] ± SE	(χ^2)[d]	*P*[e]
Beauveria bassiana	3	$3.9x10^7$ ($1.3x10^7$ - $1.5x10^8$)	$6.5x10^{12}$ ($2.1x10^{11}$ - $5.1x10^{15}$)	0.25±0.04	5.10	0.164
	5	$1.2x10^6$ ($4.0x10^5$ - $3.5x10^6$)	$4.5x10^{11}$ ($3.1x10^{10}$ - $4.3x10^{13}$)	0.23±0.03	4.12	0.389
	7	$3.0x10^5$ ($1.2x10^5$ - $6.8x10^5$)	$3.2x10^9$ ($7.4x10^8$ - $2.5x10^{10}$)	0.32±0.03	1.44	0.836
Bacillus thuringiensis	3	$2.6x10^8$ ($8.5x10^7$ - $1.3x10^9$)	$5.6x10^{13}$ ($1.4x10^{12}$ - $8.9x10^{16}$)	0.24±0.04	0.68	0.879
	5	$2.2x10^7$ ($8.9x10^6$ - $4.9x10^7$)	$2.3x10^{11}$ ($3.2x10^{10}$ - $6.3x10^{12}$)	0.32±0.04	0.62	0.893
	7	$8.2x10^6$ ($3.2x10^6$ - $1.8x10^7$)	$5.9x10^{10}$ ($1.5x10^{10}$ - $4.0x10^{11}$)	0.33±0.03	7.29	0.121

[a,b]The concentration causing 50 and 90% mortality.
[c] Slope of the concentration-mortality regression line ± standard error.
[d] Chi square value.
[e] Probability value.

7 days, respectively.

Toxicity of the three CSIs against second larval instar of *S. frugiperda* 3, 7 and 10 days after treatment expressed as LC_{50} values is summarized in Table 3. CSIs had a strong toxicity against *S. frugiperda* larvae. Hexaflumuron displayed the greatest insecticidal effect with LC_{50} values of 0.01, 0.009 and 0.005 mg/L 3, 7 and 10 days after treatment, respectively. Chlorfluazuron and lufenuron were highly effective 10 days after treatment as their LC_{50} values were 0.09 and 0.06 mg/L, respectively, while both compounds showed moderate toxicity 3 and 7 days after treatment.

The delayed effect of CSIs on pupation, emergence and malformation of pupae and adults is presented in Table 4. Pupation and adult emergence percentages of treated larvae decreased significantly with increasing concentrations of the tested CSIs compared to untreated larvae (95.0 and 94.9 %). The treatment with chlorfluazuron at 1.0 mg/L and hexaflumuron at 0.5 mg/L caused complete inhibition of pupation. Moreover, treatments with 0.25 mg/L of chlorfluazuron and hexaflumuron, and with 2.5 mg/L of lufenuron could induce complete suppression of adult emergence. Also, the treatment with the CSIs induced malformation of pupae and adults. Chlorfluazuron and lufenuron at 0.25 mg/L caused 50.0% malformation of pupae. Chlorfluazuron at 0.1 mg/L, hexaflumuron at 0.05 mg/L and lufenuron at1.0 mg/L resulted in 33.3, 25.0 and 33.0% malformation of adults, respectively.

Discussion

The insecticidal effects of spinosad, abamectin, *B. bassiana*, *B. thuringiensis*, chlorfluazuron, hexaflumuron and lufenuron have been reported against *S. frugiperda* strains present in some countries around the world (Polanczyk *et al.,* 2000; Mendez *et al.,* 2002; Eriksson, 2019; Kulye *et al.,* 2021). However, this is the first study on the toxicity of these compounds or products against *S. frugiperda* strain present in Egypt.

The treatment of castor bean leaves with spinosad and abamectin induced pronounced mortality of second larval instar of *S. frugiperda* 3 days after treatment with LC_{50} values less than 0.2 mg/L. In agreement

Table 3. Comparative toxicity of three chitin synthesis inhibitors against second instar larvae of *Spodoptera frugiperda* 3, 7 and 10 days after treatment.

Insecticide	Time (days)	LC_{50}[a] (mg/L) (Confidence limits)	LC_{90}[b] (mg/L) (Confidence limits)	Slope[c] ± SE	(χ^2)[d]	*P*[e]
Chlorfluazuron	3	0.34 (0.25-0.55)	7.81 (3.31-31.27)	0.95±0.11	3.99	0.263
	7	0.34 (0.24-0.45)	13.37 (6.36-39.0)	0.80±0.08	7.96	0.093
	10	0.09 (0.06-0.12)	7.35 (3.34-24.32)	0.66±0.07	7.44	0.114
Hexaflumuron	3	0.01 (-)	1.26 (-)	0.63±0.13	6.65	0.036
	7	0.009 (0.003-0.018)	1.09 (0.41-9.69)	0.62±0.13	4.92	0.085
	10	0.005 (-)	0.43 (-)	0.67±0.14	6.50	0.039
Lufenuron	3	0.43 (0.28-0.76)	101.18 (28.26-777.28)	0.54±0.07	2.09	0.719
	7	0.29 (0.20-0.42)	32.84 (13.51-120.87)	0.62±0.06	3.02	0.697
	10	0.06 (0.03-0.12)	85.03 (17.76-1528.62)	0.41±0.06	2.51	0.642

[a,b]The concentration causing 50 and 90% mortality.
[c] Slope of the concentration-mortality regression line ± standard error.
[d] Chi square value.
[e] Probability value.

with the present results, spinosad has been reported to induce high toxicity against larvae of *S. frugiperda* with LC_{50} of 0.557 mg/L 4 days after treatment (Hardke *et al.,* 2011). Furthermore, Adamczyk *et al.* (1999) evaluated the toxicity of spinosad against third instar larvae *S. frugiperda* and found the LC_{50} value of this compound to be 4.4 mg/L, which is similar to the obtained value (LC_{50} = 4.01 mg/L) in our study. The results may support the potential use of spinosad for the management of the fall army warm as it interferes with nicotinic acetylcholine and γ-aminobutyric acid (GABA) receptors through pathways dissimilar from those of other insecticides, i.e,. it stimulates the nervous system of insects, causing uncontrolled movement, paralysis, and death (Salgado, 1998; De Deken *et al.,* 2004). On the other hand, abamectin showed higher toxicity against the second larval instar of *S. frugiperda* in this study than that demonstrated by Ahissou *et al.* (2021) who reported an LC_{50} value of 58.5-429.9 mg/L against third instar larvae two days after treatment using the IRAC leaf bioassay protocol. These discrepancies in toxicity could be attributed to differences in insect strain, larval stage, time after treatment and assay method. Abamectin binds with γ-aminobutyric acid (GABA) receptors, leading to open chloride channel and thus allowing more chloride ions to enter the nerve cell and disturb the transportation of nerve pulses, consequently, resulting in insect paralysis and stop of feeding (Rohrer and Arena, 1995). Gutierrez-Moreno (2017) and Sisay *et al.* (2019) stated that spinosad and abamectin had the potential for the control of *S. frugiperda* in crop fields.

The *B. bassiana* strain caused high larval mortality of *S. frugiperda* 7 days after treatment with LC_{50} 3.0×10^5 spores/ml which was greater than that reported by Ramanujam *et al.* (2020) against the second larval stage

Table 4. Effect of chitin synthesis inhibitors on pupation and adult emergence of *Spodoptera frugiperda*.

Conc. (mg/L)	Pupation %	Pupal deformation %	Adult emergence %	Adult deformation %	Survival %
Chlorfluazuron					
0.0	95.0±2.1a	0.0±0.0c	94.9±2.0a	0.0±0.0b	90.0±1.0a
0.01	60.0±4.1b	8.3±0.2bc	66.6±6.7a	8.0±2.9ab	46.7±2.8b
0.05	40.0±2.0bc	12.5±1.0abc	50.0±2.1ab	25.0±1.0ab	20.0±2.5bc
0.1	26.7±5.0bc	25.0±2.0ab	50.0±7.0ab	33.3±5.8a	13.3±2.4c
0.25	20.0±2.1cd	50.0±2.1a	0.0±0.0b	0.0±0.0b	0.0±0.0d
0.50	6.7±2.4de	16.7±2.4abc	0.0±0.0b	0.0±0.0b	0.0±0.0d
1.0	0.0±0.0e	-	-	-	-
Hexaflumuron					
0.0	95.0±2.1a	0.0±0.0a	94.9±2.0a	0.0±0.0a	90.0±1.0a
0.01	40.0±1.2b	0.0±0.0a	83.3±6.0ab	16.0±3.0a	33.3±3.1b
0.05	33.3±2.3bc	25.0±2.0a	66.7±5.8ab	25.0±2.0a	20.0±1.6bc
0.1	13.3±3.1bcd	33.0±6.0a	33.0±1.2ab	16.6±3.1a	6.7±2.4cd
0.25	7.0±1.2cd	17.0±2.4a	0.0±0.0b	0.0±0.0a	0.0±0.0d
0.50	0.0±0.0d	-	-	-	-
Lufenuron					
0.0	95.0±2.1a	0.0±0.0d	94.9±2.0a	0.0±0.0a	90.0±1.0a
0.01	60.0±2.5b	8.0±3.4c	50.0±1.2b	0.0±0.0a	30.0±1.6b
0.05	53.3±4.7bc	9.7±0.9bc	44.4±3.9bc	0.0±0.0a	23.3±0.6bc
0.1	46.7±6.2bc	25.0±0.8b	38.9±2.2bc	17.0±3.1a	16.7±2.3bc
0.25	46.6±5.0bc	50.0±2.0a	16.6±1.0bcd	0.0±0.0a	5.0±0.4cd
0.50	20.0±4.1cd	0.0±0.0d	11.1±2.3bcd	0.0±0.0a	3.3±1.1d
1.0	20.0±2.0cd	0.0±0.0d	8.3±1.2cd	33.0±1.1a	1.7±0.6d
2.5	13.3±2.4d	0.0±0.0d	0.0±0.0d	0.0±0.0a	0.0±0.0d

Values in columns within each compound followed by the different letters are significantly different ($P < 0.05$).

of *S. frugiperda* (LC_{50} = 1.9 × 10^7 spores/ml). The current results are in agreement with those by Garcia *et al.* (2011) reporting that 1×10^9conidia/ml of *B. bassiana* induced 96.6% mortality on second instar larvae of *S. frugiperda*. although they did not determine LC_{50} values. Morales-Reyes *et al.* (2013) and Ramanujam *et al.* (2020) reported that mortality ranged between 10 and 65% at two concentrations of *B. bassiana* (1 × 10^6 and 1×10^7 conidia/ml). The *B. bassiana* conidia start germination when they contact insect body and germinated spores penetrate into insect body via cuticle joints. After penetration inside the insect`s body, the fungus starts to invade other insect tissues and continues a vegetative growth producing toxic compounds, eventually leading to insect`s death (Logrieco *et al.,* 2002).

Bacillus thuringiensis is among the most used microbial biopesticides for Lepidoptera pest control due to its high efficacy and less adverse effects on mammals and non-target organisms. In the current study, *B. thuringiensis* showed a pronounced toxicity against *S. frugiperda* larvae with an LC_{50} value of 8.2 × 10^6 cells/ml after 7 days. Similar results against the second larval stage of *S. frugiperda* were found by Polanczyk *et al.* (2000) for *B. thungiensis* at LC_{50} value as 8.6 × 10^6 cells/ml. Hernandez (1988) stated that *B. thuringiensis kurstaki* caused 70% mortality of the second larvae stage of *S. frugiperda* at a concentration of 3 × 10^7 cells/ml. Capal-

bo *et al.* (2001) found that the application of *B. thuringiensis* against *S. frugiperda* achieved complete mortality of neonate larvae in field trials. Furthermore, other strains of *B. thuringiensis* have been shown to possess toxicity against *S. frugiperda* larvae (Dos Santos *et al.,* 2009; Loto *et al.,* 2019; Varshney *et al.,* 2021). A protoxin large protein (about 130-140 kDa) of *Bt* solubilises in the insect gut and cleaves by a gut protease to yield a delta-endotoxin (about 60kD) which binds to the midgut epithelial cells, making openings in the membranes and resulting in an equilibration of ions. Consequently, the gut is quickly immobilised, the epithelial cells lyse, the larva stops feeding, and the gut pH is dropped by equilibration with the blood pH. This lower pH allows the bacterial spores to sprout, and the bacterium can then invade the host, inducing a lethal septicaemia (Sanchis and Bourguet, 2008; Schünemann *et al.,* 2014).

The CSIs induced strong toxicity against *S. frugiperda* second instar larvae with hexaflumuron being more effective than chlorfluazuron and lufenuron. The LC_{50} value of lufenuron was 0.29 mg/L 7 days after treatment, which was similar to that (LC_{50} = 0.23 mg/L) obtained by Nascimento *et al.* (2016). But lower than that reported by Eriksson (2019) (LC_{50} = 0.12 mg/L) on the third larval stage 4 days after treatment. Moreover, novaluron has shown high toxicity against *S. frugiperda* larvae with LC_{50} value (0.166 mg/L) (Hardke *et al.*, 2011). Beside their effect on larval mortality, the tested CSIs (chlorfluazuron, hexaflumuron and lufenuron) induced significant malformation in pupae and adults, and reduced adult emergence. These results coincide with the results of earlier studies on the activity of CSIs against lepidopteran insects (Whiting *et al.*, 2000; Butter *et al.,* 2003; Biddinger *et al.*, 2006). The recorded malformation effect and inhibition of adult emergence of CSIs are probably due to their inhibitory effects on the chitin synthesis, which adversely affect insect metamorphosis (Khajepour *et al.*, 2012; Hamadah *et al.*, 2015). CSIs inhibit chitin formation in the procuticle and the deposition of epicuticle, causing and unsuccessful molt and decease. Likewise, CSIs have been confirmed to decrease egg fertility and hatching (Haroardottir *et al.*, 2019).

Conclusion

Based on the outcome of the present study, the non-conventional insecticidal substances, spinosad, abamectin, *B. bassiana, B. thuringiensis*, chlorfluazuron, hexaflumuron and lufenuron, revealed a promising toxicity against *S. frugiperda* larvae in Egypt with hexaflumuron being the most effective one in terms of LC_{50}. Therefore, these products may be useful for the IPM management of this invasive insect. The use of such products with diverse mechanisms of action is highly important to delay the development of insect resistance. Also, the use of naturally based products is expected to minimize the impact on non-target organisms, mammals and the environment.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Συγκριτική δράση βιοεντομοκτόνων κατά του εντόμου *Spodoptera frugiperda* (J.E. Smith)

A.A.M. Atta, A.A. Al-Ayat, H.A. Gad και S.A.M. Abdelgaleil

Περίληψη Η παρούσα εργασία εξέτασε τη δράση εντομοκτόνων μικροβιακής προέλευσης (abamectin, spinosad), δύο μικροοργανισμών (*Beauveria bassiana* (Balsamo) Vuillemin, *Bacillus thuringiensis* Berliner) και τριών αναστολέων σύνθεσης χιτίνης (CSIs) (chlorfluazuron, hexaptenauruguronper, spofendoflumuron) σε προνύμφες του εντόμου *Spodoptera frugiperda*. Το spinosad και η abamectin προκάλεσαν μεγάλη θνησιμότητα έναντι του δεύτερου προνυμφικού σταδίου του *S. frugiperda* μετά από έκθεση με τη μέθοδο της εμβάπτισης φύλλων. Το spinosad προκάλεσε υψηλότερη τοξικότητα (LC_{50} = 4,01 mg/L) από την abamectin (LC_{50} = 8,33 mg/L), μία ημέρα μετά την εφαρμογή. Οι επεμβάσεις με *B. bassiana* και *B. thuringiensis* προκάλεσαν υψηλότερη θνησιμότητα των προνυμφών του *S. frugiperda*, επτά ημέρες μετά την εφαρμογή, με τιμές LC_{50} $3,0 \times 10^5$ σπόρια/ml και $8,2 \times 10^6$ σπόρια/ml, αντίστοιχα. Στην περίπτωση των CSI, το hexaflumuron έδειξε υψηλότερη τοξικότητα από το chlorfluazuron και το lufenuron με τιμές LC_{50} 0,01, 0,009 και 0,005 mg/L, 3, 7 και 10 ημέρες μετά την εφαρμογή, αντίστοιχα.

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Antibacterial potential of extracts and metabolites isolated from the endophytic fungus *Chaetomium cochliodes* against phytopathogenic bacteria

M.M.G. Saad[1] and S.A.M. Abdelgaleil[1]*

Summary Five fungal endophytes, *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Rhizopus* sp. and *Curvularia* sp., were isolated from an Egyptian herbaceous plant, *Tribulus terrestris*, and tested for their antibacterial activity against three phytopathogenic bacteria (*Pectobacterium carotovorum* subsp. *carotovorum, Ralstonia solanacearum, Pseudomonas syringae* pv. *syringae*). *Chaetomium* sp. showed the highest antibacterial activity. This strain was identified morphologically and molecularly as *Chaetomium cochliodes* MS03 (MW898133) based on the ITS1-5.8S rRNA-ITS2 genomic region. *Chaetomium cochliodes* caused 15 and 8 mm inhibition zones of *P. carotovorum* subsp. *carotovorum* and *R. solanacearum*, respectively. *Chaetomium cochliodes* isolate was fermented and extracted with ethyl acetate. The crude extract of *C. cochliodes* showed strong antibacterial activity against *P. carotovorum* subsp. *carotovorum* (inhibition zone = 27 mm). Bioassay guided isolation of the crude extract using silica gel column chromatography was conducted to isolate bioactive secondary metabolites. Minimum inhibitory concentrations (MICs) were 500, 32 and 4 mg/L for *C. cochliodes* extract, fraction 14 and fraction 15, respectively, against *P. carotovorum* subsp. *carotovorum*. Bioactive fractions were analyzed by GC/MS. The bioactive pure compound was identified as 9,12-octadecadienoic acid (Z,Z) and the chemical structure was confirmed by H^1 NMR and C^{13} NMR spectral analysis. The isolated compound showed a promising antibacterial activity against *P. carotovorum* subsp. *carotovorum* with MIC value of 32 mg/L.

Additional keywords: antibacterial activity, endophytic fungi, secondary metabolites, structure identification, *Tribulus terrestris*

Introduction

The interaction between microorganisms and their hosts is liable to the nature of the host and the surrounding environment; it can be symbiotic or mutualistic or pathogenic (Sahani and Hemalatha, 2018). Fungi are good examples of these relationships, they can be found as plant pathogens, and/or they can live as endophytes, asymptomatically without causing any signs of diseases, within the intracellular spaces of leaves, roots and stem tissues (Arnold *et al.,* 2000). When numerous microbial species exist in the same plant, their interaction may promote the secretion of some secondary metabolites by the endophytes or the host that obstruct the development of the harmful microbes (Kusari *et al.,* 2012).

Several endophytes are known for their ability to improve nutrient acquisition, promote growth, increase abiotic and biotic stress tolerance of the host plant, and enhance plant defense against many phytopathogens. Thus, endophytes are considered as efficient bio-control agents (Saad and Badry, 2020). Likewise the extracellular secondary metabolites produced by endophytic fungi occasionally possess biological activity against various plant pathogens (Mousa and Raizada, 2013). Different bioactive compounds with antimicrobial properties have been isolated from endophytes, such as alkaloids, flavonoids, peptides, polyketides, phenols, steroids, terpenoids, and quinones (Gunatilaka, 2006; Mousa and Raizada, 2013; Lugtenberg *et al.,* 2016).

Fatty acids are long, unbranched carbon chain carboxylic acids with saturated or unsaturated bonds. Fatty acids and their deriv-

[1] Department of Pesticide Chemistry and Technology, Faculty of Agriculture, El-Shatby, Alexandria University, Alexandria 21545, Egypt.
* Corresponding author: samirabdelgaleil@gmail.com

atives are major components of plant and fungal metabolites. They are playing a crucial role in host defense against pests having a broad potential of antibacterial (Kabara *et al.,* 1972), antifungal (Walters *et al.,* 2004), antimalarial (Carballeira, 2008), antifeedant, insecticidal and nematicidal (Stadler *et al.,* 1994) activities. Therefore, there is a continuous research interest in isolating natural compounds from endophytes which could serve as alternatives to synthetic pesticides.

Pectobacterium carotovorum subsp. *carotovorum* is a phytopathogenic, soil borne facultative anaerobic bacterium causing soft rot, blackleg or stem rot in many economically important crops, including vegetables, ornamental plants and fruits (Pérombelon and Kelman, 1980). Soft rot is a worldwide distributed disease affecting a variety of vegetable crops, such as potatoes (*Solanum tuberosum*), Chinese cabbage (*Brassica pekinensis*), carrots (*Daucus carota*), etc. It causes severe disease to vegetables during cultivation, post-harvest handling, and storage (Strange and Scott, 2005). *Ralstonia solanacearum* is a soil-borne bacterium causing the widespread disease known as bacterial wilt (Peeters *et al.*, 2013). *Pseudomonas syringae* pv. *syringae* constitutes a diverse group of bacterial strains that cause important diseases, such as bacterial canker of stone fruits, citrus blast, leaf blight of wheat and barley, sheath rot of rice, red streak of sugarcane and brown spot of bean (Bultreys and Kaluzna 2010; Dariush *et al.,* 2012).

Synthetic antibiotics, such as kasugamycin, gentamicin, streptomycin, oxolinic acid, oxytetracycline and validamycin are used to control pathogenic bacteria (Verhaegen *et al.*, 2024). However, due to their environmental hazards, their side effects on non-target organisms and the development of pathogen resistance, their use has become under restrictions. Thus the continuous efforts for discovering and developing new antimicrobial compounds from natural sources, including endophytic fungi, to overcome these difficulties, are very crucial and ever-increasing.

The present research aims to isolate and investigate the potential of endophytic fungi as a source of natural pesticides, focusing on endophytic fungi in plants native to Egypt for evaluation of their potential antibacterial activity, isolation and identification of the compounds responsible for such antibacterial activity. Thus, the isolation and the antibacterial activity assessment of five fungal isolates obtained from *Tribulus terrestris* L. (Zygophyllaceae), a plant known in ancient medicine for its diuretic, tonic, and aphrodisiac properties, were carried out. In addition, the antibacterial activity of extract, fractions and a pure compound from one of these fungi, *C. cochliodes*, was evaluated against *P. carotovorum* subsp. *carotovorum*.

Materials and methods

Sampling and isolation of endophytes

Apparently healthy and fresh leaves from specimens of *Tribulus terrestris* were collected from Shalalat garden, Alexandria (31°12′56.30″N, 29°57′18.97″E), Egypt. The plant was identified by Prof. FathAllah Zaitoon of Department of Plant Pathology, University of Alexandria. Fungal endophytes isolation process was performed within 24 h of sampling by a standardized surface sterilization method. Briefly, plant leaves were washed in running tap water, immersed in 70% ethanol for 1 min, then in 3% sodium hypochlorite solution for 2 min, and rinsed in sterile distilled water three times separately. The surface sterilized leaves were cut into 5 mm pieces with a sterile blade, inoculated onto Petri plates containing Potato Dextrose Agar (PDA) supplemented with chloramphenicol (100 mg/L), incubated at 28°C ± 2 for 14 days in the dark and checked every other day for emerging hyphae. Within the first week, the emerging hyphae were transferred to fresh PDA plates for sub-culturing several times to ensure pure isolates.

Morphological and molecular identification of fungal endophytes

Fungal cultures were maintained on PDA

at 28°C ± 2°C in the dark for 14 days, then visually examined for their morphological characterization to genus level based on macroscopic and microscopic features, such as colony color, growth rate, type of conidiophore and shape of conidia (Barnett and Hunter, 1998). Five fungal isolates (*Alternaria* sp.*, Aspergillus* sp.*, Chaetomium* sp.*, Rhizopus* sp. and *Curvularia* sp.) were identified and examined for their antibacterial activity as described in the following paragraph. The isolate (*Chaetomium* sp.) showing the highest activity was subjected to molecular identification to species level and phylogenetic analysis. Molecular identification using ITS-PCR amplification was conducted from a one-week-old fungal isolate in PDA culture. The fungal strain identification was performed based on sequencing analysis of the amplified ITS1-5.8S rRNA-ITS2 genomic region (White *et al.,* 1990). The amplified PCR product of ITS1- 5.8SrDNA-ITS2 was sequenced on both strands using the primer set: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), an automated ABI-Prism 377 DNA Sequencer (Applied Biosystems Inc., CA, USA) and a Taq FS Dye Terminator Sequencing Kit (ABI, USA). Sequence editing was carried out using Biology Work Bench 3.7 software. The sequence was compared to the available fungal sequences on the NCBI database using the Blast program (http://www.ncbi.nlm.nih.gov/genbank/) and the accession numbers were obtained. This was achieved by generating a neighbor-joining distance-based tree using the software MEGA 6.

Antagonistic effects of the fungal isolates against phytopathogenic bacteria

Three phytopathogenic bacterial strains, *P. carotovorum* subsp. *carotovorum* (Jones, 1901) Hauben *et al.* 1999 (EMCC 1687), *P. syringae* pv. *syringae* Van Hall, 1904 (EMCC 1739), and *R. solanacearum* (Smith, 1896) Yabuuchi *et al.,* 1996 (EMCC 1274) were obtained from Microbiological Resource Centre (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The bacterial strains were cultured in Nutrient broth (NB) overnight at 30°C and their concentration was adjusted to 10^8 CFU ml^{-1}. The antagonistic activities of the five isolated endophytic fungi were tested as mycelium on agar plates against the three selected phytopathogenic bacteria, and were assessed by measuring the inhibition zone diameter. One milliliter of bacterial culture was scattered evenly onto nutrient agar plates using a sterile Drigalsky's handle, then a disk of 5 mm Ø of seven day-old mycelia of each endophytic fungus was placed in the central of the NA plate. Plates were incubated for approximately 24 h at 30°C and the diameter of the inhibition zones were measured in mm.

Fermentation and extraction of metabolites

Solid-state fermentation was carried for the most active antibacterial fungal strain isolated in this study, *Chaetomium cochliodes* (MW898133, as registered in NCBI, in this study). A pure colony of this isolate was inoculated in 1 L Erlenmeyer flasks containing autoclaved barley medium. For the barley medium, barley (100 g) was soaked in 150 mL of distilled water overnight then autoclaved at 121°C and pressure of 15 psi for 20 minutes. The inoculated flasks were incubated with shaking at 28°C for 30 days. Then the fungal *hyphae* and growth medium were extracted with ethyl acetate (EtOAc) and the extract was concentrated under vacuum. This crude extract was kept at 4°C for further investigations.

Primary antimicrobial evaluation of *C. cochliodes* extract

Preliminary antimicrobial screening of the *C. cochliodes* crude extract was carried out using the agar well diffusion method. The extract was tested against the *P. carotovorum* subsp. *carotovorum* strain EMCC 1687. Nutrient agar was poured into sterile Petri dishes and after solidification standardized concentration of an overnight culture of the *P. carotovorum* subsp. *carotovorum* strain was swabbed aseptically on the agar. Holes (5 mm diameter) were made in the agar plates using a sterilized cork bor-

er. Fifty microliters of entophyte extract solution (1000 mg/L) and DMSO (negative control) were put in each hole. Three replicates of treatment (endophyte extract) and control were arranged in each plate. The plates were incubated at 30°C for 24 hours and the inhibition zone diameters were measured.

Isolation of bioactive compound from fungal extract

Column fractionations of the EtOAc crude extract (2 g) were conducted using silica gel column chromatography two times. The first fractionation of the crude extract with column 3×50 cm filled with 70 g silica gel (200-300 mesh) and eluted with 1L of methylene chloride- methanol (0%, 0.25%, 0.5%, 1% and 5% MeOH/ CH_2Cl_2 v/v) to give fractions (1–15). The antibacterial activities of the 15 column fractions were tested using the 96-well plate bioassay. Fraction 14 (F14) (1% MeOH/CH_2Cl_2, 300 mg), which showed the highest activity followed by fraction 15 (F15), was subjected to further purification on silica gel column chromatography (1× 50 cm) eluted with hexane-ethyl acetate (100:0 – 0:100 v/v) to give a pure compound (170 mg).

GC/MS analysis of the bioactive fractions

The chemical composition analysis of the most bioactive column fractions (F14 and F15) was performed using Trace GC-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was initially held at 50°C and then increased to 250°C by 5°C /min and held for 2 min. The temperature was increased to 300°C by 30°C /min and held for 2 min. The temperatures of the injector and MS transfer line were kept at 270 and 260°C respectively. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 4 min and diluted samples of 1 µl were injected automatically using Auto sampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 50–650 in full scan mode. The ion source temperature was set at 200°C. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 14 mass spectral database.

NMR of the isolated compound

The NMR spectra of the isolated compound, 9,12-octadecadienoic acid (Z,Z), were obtained on a Bruker BioSpin GmbH spectrometer (400 MHz). A sample (10 mg) of the compound was dissolved in 0.5 ml of $CDCl_3$. The ^{1}H and ^{13}C NMR spectra were taken at 25°C, and the chemical shift was expressed in parts per million.

Determination of minimum inhibitory concentrations (MICs) using micro-dilution assay

The antimicrobial activity of *C. cochliodes* crude extract, the column fractions and the isolated compound were tested in 96-well plates using nutrient broth as culture medium to determine the minimum inhibitory concentration (MIC). The bacterial inoculum of *P. carotovorum* subsp. *carotovorum* was prepared as described earlier. Stock solutions of the tested crude extract, the column fractions (15 fractions) and the isolated compound were prepared in dimethyl sulfoxide (DMSO). Apposite volumes of the stock solutions were transferred to 96-well plates contained the appropriate amount of NB to obtain final concentrations of 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8 and 3.9 mg/L. Then the bacterial inoculum (1.0×10^8 CFU ml^{-1}) was added and the 96-well plates (micro-dilution trays) were incubated at 30°C. After 24 h of incubation 20 µl of 2,3,5-triphenyltetrazolium chloride (TTC) was added and incubated for 30 min. at 30°C in the dark (Ellof, 1998). A change of color from colorless to pink indicated the reduction of TCC by the viable bacterial cells. The MIC was defined as the lowest concentration of the tested compounds that prevented this color change. DMSO was used as negative control and a reference antibiotic, Ampicillin, was

used as positive control.

Statistical analysis

Experimental results (diameter of inhibition zone) were the average values of three replicates. For the analysis of the data obtained from the fungal mycelial disk diffusion assay, data was subjected to one-way analysis of variance followed by Student-Newman Keuls test to determine significant differences between mean values at the probability level of 0.05.

Results

Isolation, morphological and molecular identification of endophytic fungi

A total of five fungal endophytes were isolated from healthy leaves of *T. terrestris*. which, after identification to the genus level (taxonomical morphological features including colony color, growth rate, type of conidiophore and shape of conidia), were classified in five genera: *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Rhizopus* sp. and *Curvularia* sp. One of these strains, that was later determined as the most bioactive antibacterial strain against *P. carotovorum* subsp. *carotovorum*, was morphologically identified as *Chaetomium* sp. and was subjected to molecular identification. The fungal isolate was molecularly identified as *C. cochliodes* based on the ITS1-5.8S rRNA-ITS2 genomic region. The acquired sequence was submitted to the NCBI GenBank database and an accession number was obtained for *C. cochliodes* MS03 (MW898133). The sequence was analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The sequence was aligned using Align Sequences Nucleotide BLAST. The identification of the species was determined based on the best sequence alignment score. The DNA sequence was included in a phylogenetic study by means of comparative sequence analysis of the other rDNA sequences (Fig. 1).

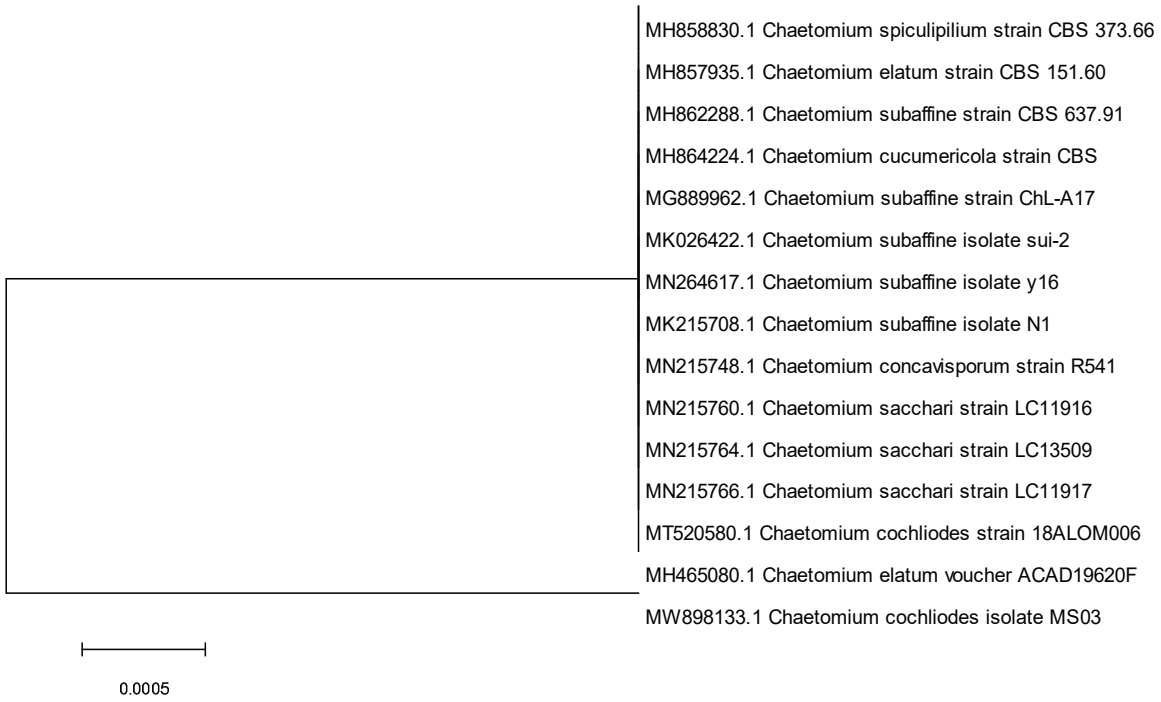

Figure 1. A neighbor-joining phylogenetic tree was constructed based on the alignment of the ITS1-5.8S rRNA-ITS2 genomic region sequences of *Chaetomium* genotypes derived from NCBI using MEGA 6 software.

Letters and numbers written in front of the scientific name are the GenBank accession numbers.

Antibacterial activities of endophytic fungi and *C. cochliodes* crude extract

The antagonistic activity of the five isolated endophytic fungi against the three selected phytopathogenic bacteria, based on the inhibition zone diameter on agar plates is presented in Table 1. *Chaetomium cochliodes* (MW898133) was the most active isolate against *P. carotovorum* subsp. *carotovorum* and *R. solanacerum* with 15 and 8 mm inhibition zones, respectively. In addition, *Aspergillus* sp. isolate showed weak antibacterial activity against *R. solanacerum* with 3 mm inhibition zone. All of the isolated endophytic fungi were not active against *P. syringae* pv. *syringae*. Based on the above-mentioned results, the EtOAc crude extract of *C. cochliodes* (MW898133) was tested for antibacterial activity against *P. carotovorum* subsp. *carotovorum* using the agar well diffusion method. This extract showed promising activity with inhibition zone 27 mm (Table 1).

GC-MS analysis of the crude extract of *C. cochliodes*

The GC–MS analysis of the most bioactive column fractions of the *C. cochliodes* (MW898133) crude extract led to the identification of nine (9) compounds in F14 and five (5) compounds in F15. The characterization of the compounds structures was obtained by comparison of mass spectra with the spectra present in those of WILEY 09 and NIST 14 mass spectral database. The characterized compounds and their retention time (RT), concentration (peak area %), molecular formula and molecular weight (MW) are presented in Tables 2 and 3.

Identification of a pure antibacterial compound

Fraction 14 of *C. cochliodes* extract that showed the highest antibacterial activity was further identified by using H^1NMR, C^{13}NMR spectral data and GC–MS as 9,12-octadecadienoic acid (Z,Z) (Fig. 2). Here are ^{1}H NMR and ^{13}C NMR spectral data of 9,12-octadecadienoic acid (Z,Z) ; ^{1}H NMR ($CDCl_3$): δ (ppm) 0.82 (3H, t, J = 7.2 Hz, Me-18), 1.18-1.25 (14H, m, H-4, H-5, H-6, H-7, H-15, H-16, H-17), 1.56 (2H, t, J = 6.4 Hz, H-3), 1.97 (2H, t, J = 7.2 Hz, H-14), 2.28 (2H, t, J = 7.2 Hz, H-2), 2.70 (2H, t, J = 6.0, H-11), 5.27-5.29 (4H, m, H-9, H-10, H-12, H-13); ^{13}C NMR ($CDCl_3$): δ (ppm) 14.1 (q, Me-18), 22.6 (t, C-17), 24.6 (t, C-3), 26.6 (t, C-11), 27.2 (t, C-8, C-14), 28.9-29.8 (t, C-4, C-5, C-6, C-7, C-15), 31.5 (t, C-16), 34.0 (t, C-2), 128.1, 128.8 (d, C-10, C-12), 130.0, 130.2 (C-9, C-13), 179.8 (s, C-1).

Antibacterial activity of *C. cochliodes* extract, fractions and isolated compound

Minimum inhibitory concentrations (MIC) of *C. cochliodes* crude extract, fractions and the isolated compound, 9,12-octadecadienoic acid (Z,Z), are shown in Table 4. The

Table 1. Antibacterial activity (zone of inhibition, mm) of five endophytic fungi and *Chaetomium cochliodes* crude extract.

Treatment	Inhibition zone (mm)		
	Ralstonia solanacerum	*Pectobacterium carotovorum* subsp. *carotovorum*	*Pseudomonas syringae* pv. *syringae*
Alternaria sp.	0.00 c*	0.00 d	0.00
Aspergillus sp.	3.00 b	7.00 c	0.00
Chaetomium cochliodes	8.00 a	15.00 b	0.00
Rhizopus sp.	0.00 c	8.00 c	0.00
Curvularia sp.	0.00 c	0.00 d	0.00
Chaetomium cochliodes extract	-	27.00 a	-
DMSO (control)	0.00 c	0.00 d	0.00

* Different letters indicate significant differences among treatments within the same column according to least significant difference test (P ≤ 0.05).

Table 2. GC/MS analysis of fraction14 separated from ethyl acetate extract of the endophytic fungus *Chaetomium cochliodes* (MW898133).

No.	Retention time	Name	Area (%)	Molecular formula	Molecular weight
1	19.74	Butylated Hydroxytoluene	2.33	$C_{15}H_{24}O$	220
2	25.07	2H-Pyran-3-ol, tetrahydro-2,2,6-trimethyl-6-(4-methyl-3-cycloh exen-1-yl)-, [3S [3à,6à(R)]]-	1.03	$C_{15}H_{26}O_2$	238
3	28.67	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8 dione	0.67	$C_{17}H_{24}O_3$	276
4	29.63	n-Hexadecanoic acid	2.34	$C_{16}H_{32}O_2$	256
5	31.28	Palmitic Acid, TMS derivative	70.22	$C_{19}H_{40}O_2Si$	328
6	32.07	Methyl 9-cis,11-trans-octadecadienoate	1.80	$C_{19}H_34O_2$	294
7	32.94	9,12-Octadecadienoic acid (Z,Z)	15.41	$C_{18}H_{32}O_2$	280
8	34.25	9,12-Octadecadienoic acid (Z,Z)-trimethylsilyl ester	4.10	$C_{21}H_{40}O_2Si$	352
9	34.34	9-Octadecenoic acid, (E)-, TMS derivative	1.21	$C_{21}H_{42}O_2Si$	354

Table 3. GC/MS analysis of fraction 15 separated from ethyl acetate extract of the endophytic fungus *Chaetomium cochliodes* (MW898133).

No.	Retention time	Name	Area (%)	Molecular formula	Molecular weight
1	19.74	Butylated hydroxytoluene	1.19	$C_{15}H_{24}O$	220
2	31.28	Palmitic acid, TMS derivative	80.86	$C_{19}H_{40}O_2Si$	328
3	32.94	9,12-Octadecadienoic acid (Z,Z)	11.17	$C_{18}H_{32}O_2$	280
4	34.25	9,12-octadecadienoic acid (Z,Z)-, trimethylsilyl ester	5.23	$C_{21}H_{40}O_2Si$	352
5	34.34	9-Octadecenoic acid, (E)-, TMS derivative	1.55	$C_{21}H_{42}O_2Si$	354

crude extract displayed the lowest antibacterial activity with MIC value of 500 mg/L. The two fractions: F14 and F15 showed strong antibacterial activity with MIC values of 32 and 4 mg/L, respectively. Fraction 15 (MIC = 4 mg/L) was more active than a reference antibiotic, ampicillin (MIC = 16 mg/L). Furthermore, the pure compound, 9,12-octadecadienoic acid (Z,Z), revealed a promising antibacterial activity as its MIC value was 32 mg/L.

Discussion

Medicinal plants have been recognized as a rich source of endophytes with potential novel secondary metabolites of agricultural and pharmaceutical merit (Tan and Zou, 2001; Strobel *et al.,* 2004). In this study, five fungal endophytes, *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Rhizopus* sp. and *Curvularia* sp., have been isolated, for the first time, from the leaves of a well-known medicinal herb, *Tribulus terrestris* growing in Egypt. In agreement with our results, Sahani and Hemalatha (2018) isolated a total of 54 endophytic isolates including *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., and *Curvularia* sp. from different parts of these plants growing in Bengal. Similarly, the isolation of two fungal strains, *Curvularia aeria* MTCC-12847 and *Alternaria tenuissima*, have been previously reported from the leaf and stem of *T. terrestris* growing in Bengal and China, respectively (Wu *et al.,* 2014; Sahani *et al.,* 2019).

Of all isolated fungal endophytes, the

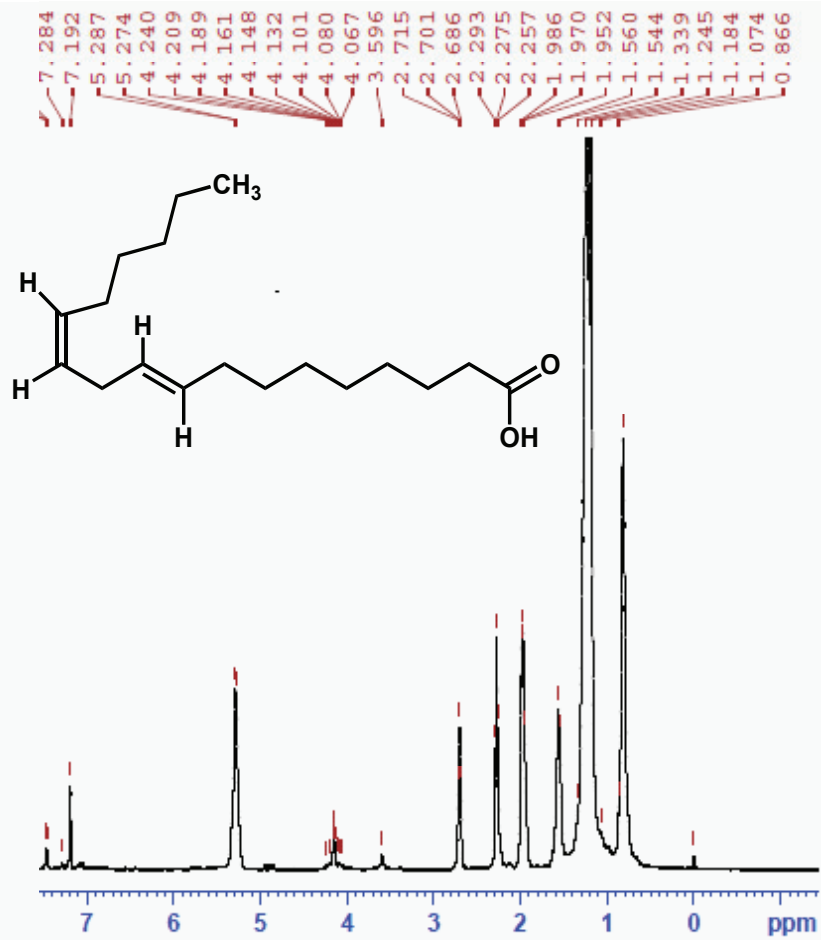

Figure 2. Chemical structure and ^{1}H NMR spectrum of 9,12-octadecadienoic acid (Z,Z) isolated from *C.haetomium cochliodes* (MW898133).

Table 4. Minimum inhibitory concentration (MIC) by the 96-well plate assay of *Chaetomium cochliodes* crude extract, column fractions and a pure isolated compound (µg/mL) against *Pectobacterium carotovorum* subsp. *carotovorum*.

Extract/compound	Minimum inhibitory concentration (MIC) mg/L
Chaetomium cochliodes extract	500
F14	32
F15	4
9,12- Octadecadienoic acid (Z,Z)	32
Ampicillin	16

extract, fractions and the isolated secondary metabolite from the endophytic fungus *C. cochliodes* (MW898133) showed strong antibacterial activity against *P. carotovorum* subsp. *carotovorum*. Similarly to our results, several fungal endophytes are known for their capacity to inhibit bacterial growth and produce compounds that have antibacterial activity (Hardoim *et al.,* 2015). For example, altersetin, an alkaloid isolated from the endophyte *Alternaria* spp., has been shown to display a strong antibacterial effect against many Gram positive bacteria (Hellwig *et al.,* 2002). An endophytic fungus, *Muscodor albus*, was reported to produce volatile compounds, such as aciphyllene, 2-butanone

and 2-methyl furan with antibiotic properties (Atmosukarto *et al.,* 2005). Furthermore, several endophytic fungi, such as *Pestalotiopsis mangiferae*, *Aspergillus* sp., *Nigrospora sphaerica* (URM-6060), *Pestalotiopsis maculans* (URM-6061) *Phomopsis* sp. and *Botryosphaeria* sp., have been described to produce secondary metabolites displaying antibacterial activity (Phongpaichit *et al.,* 2006; Pinheiro *et al.,* 2013; Subban *et al.,* 2013; Santos *et al*., 2015).

Regarding the presence of fatty acids in the *C. cochliodes* extract, fatty acids and their derivatives have been isolated by bioassay-guided fractionation from numerous plants and organisms as protectants against pathogenic bacteria (Han *et al.,* 2003; Desbois *et al.,* 2009 and 2010; Tanvir *et al.,* 2017 and 2018). According to Sumayo *et al*. (2014), linoleic acid was found to elicit induced systemic resistance (ISR) of tobacco against the bacterial soft rot pathogen, *P. carotovorum* subsp. *carotovorum*. Hexadecanoic acid ethyl ester showed antioxidant, nematicidal, pesticidal and antimicrobial activities (Farmer and Ryan, 1992; Blechert *et al.,* 1995). There are prior reports indicating the presence of fatty acids in endophytic fungi extracts and these fatty acids had been proven to be widely bioactive against gram-positive and gram-negative bacteria (Han *et al.,* 2003; Smith *et al.,* 2015; Malhadas *et al.,* 2017). Manganyi *et al*. (2019) stated that among 133 endophytic fungal strains isolated from *Pelargonium sidoides* only the isolate MHE 68, identified as *Alternaria* sp., had antibacterial activities against clinical bacteria strains, *Enterococcus faecium* and *E. gallinarum*. The chemical analysis of this *Alternaria* sp. extract indicated the presence of 9,12-octadecadienoic acid (Z,Z) and cyclodecasiloxane which, according to authors, could be accountable for the antibacterial activity.

Furthermore, many studies have exposed a relationship between the structure of fatty acids and their antimicrobial properties; unsaturated fatty acids are more effective than saturated fatty acids and the double bonds position is substantial for long chain fatty acids. (Kurihara *et al.,* 1999; Cerdeiras *et al.,* 2000; Zheng *et al.,* 2005; Won *et al.,* 2007; Hamel, 2009; Sado-Kamdem *et al.,* 2009). Likewise, our findings indicated that the most active fractions against *P. carotovorum* subsp. *carotovorum* were those containing the unsaturated fatty acids and their derivatives, while the fractions which contain saturated fatty acids were less active. This could also extend to the presence of methyl 9-*cis*,11-*trans*-octadecadienoate in F14, as the F14 had a more potent antibacterial activity compared to F15 that lacks methyl 9-*cis*,11-*trans*-octadecadienoate, given that esters have a remarkable effect on antimicrobial activity. The stereochemistry of unsaturated compounds has an important role in bioactivity as *cis*-isomers are more active than *trans*-isomers probably because the structures of *trans*-bonded unsaturated fatty acids resemble to the saturated acids and this was noticeable in our study since the active compound was a *cis* isomer (Wille and Kydonieus, 2003; Desbois and Smith, 2010).

Despite that the antibacterial mode of action of fatty acids is still poorly understood, several studies suggested that their prime target is the cell membrane; they disrupt the electron transport chain and oxidative phosphorylation. They also interfere with cellular energy production, inhibit enzyme activity, reduce nutrient uptake, causing peroxidation and auto-oxidation degradation or lysis of bacterial cells (Kurihara *et al.,* 1999; Zheng *et al.,* 2005; Won *et al.,* 2007; Hamel, 2009; Kenny *et al.,* 2009; Sado-Kamdem *et al.,* 2009).

In conclusion, the present study provides information on the isolation, identification and antagonistic activities of the five endophytic fungi (*Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Rhizopus* sp. and *Curvularia* sp.), as mycelia, against three phytopathogenic bacteria (*P. carotovorum* subsp. *carotovorum*, *R. solanacearum* and *P. syringae* pv. *syringae*) with *Chaetomium* sp. being the most active against *P. carotovorum* subsp. *carotovorum*, *R. solanacearum*. In addition, the extract, fractions and a fatty acid, 9,12-octadecadienoic acid (Z,Z), isolat-

ed from *C. cochliodes* (MW898133) showed promising antibacterial activity against *P. carotovorum* subsp. *carotovorum*. The findings encourage further studies on the antibacterial mode of action and safety of endophytic fungi extracts and their secondary metabolites as a renewable source of bioactive compounds with possible application in agriculture and medicine.

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Αντιβακτηριακή δράση εκχυλισμάτων και μεταβολιτών που απομονώθηκαν από τον ενδοφυτικό μύκητα *Chaetomium cochliodes* έναντι φυτοπαθογόνων βακτηρίων

M.M.G. Saad και S.A.M. Abdelgaleil

Περίληψη Πέντε ενδοφυτικοί μύκητες, *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Rhizopus* sp. και *Curvularia* sp., απομονώθηκαν από το Αιγυπτιακής προέλευσης ποώδες φυτό *Tribulus terrestris*, και ελέγχθηκαν ως προς την αντιβακτηριακή τους δράση έναντι τριών φυτοπαθογόνων βακτηρίων (*Pectobacterium carotovorum* subsp. *carotovorum, Ralstonia solanacearum, Pseudomonas syringae* pv. *syringae*). Ο μύκητας *Chaetomium* sp. παρουσίασε τη μεγαλύτερη αντιβακτηριακή δράση. Το στέλεχος αυτό αναγνωρίστηκε μορφολογικά και μοριακά ως *Chaetomium cochliodes* MS03 (MW898133) με βάση τη γονιδιωματική περιοχή ITS1-5.8S rRNA-ITS2. Ο μύκητας *C. cochliodes* προκάλεσε ζώνες αναστολής ανάπτυξης 15 και 8 mm του *P. carotovorum* subsp. *carotovorum* και του *R. solanacearum*, αντίστοιχα. Μετά από διεργασία ζύμωσης με τον μύκητα *C. cochliodes*, πραγματοποιήθηκε εκχύλιση με οξικό αιθυλεστέρα. Το ακατέργαστο εκχύλισμα του *C. cochliodes* έδειξε ισχυρή αντιβακτηριακή δράση έναντι του *P. carotovorum* subsp. *carotovorum* (ζώνη αναστολής ανάπτυξης= 27 mm). Βιοδραστικοί δευτερογενείς μεταβολίτες απομονώθηκαν από το ακατέργαστο εκχύλισμα με στήλη χρωματογραφίας διοξειδίου του πυριτίου (silica gel) και βιοδοκιμή. Οι ελάχιστες συγκεντρώσεις αναστολής της ανάπτυξης ήταν 500, 32 και 4 mg/L για το εκχύλισμα του *C. cochliodes*, το κλάσμα 14 και το κλάσμα 15, αντίστοιχα, έναντι του *P. carotovorum* subsp. *carotovorum*. Τα βιοδραστικά κλάσματα αναλύθηκαν με GC/MS. Η βιοδραστική καθαρή ένωση ταυτοποιήθηκε ως 9,12-οκταδεκαδιενοϊκό οξύ (Z, Z) και η χημική δομή επιβεβαιώθηκε με φασματική ανάλυση H^1 NMR και C^{13} NMR. Η ένωση που απομονώθηκε έδειξε ενθαρρυντικά αποτελέσματα για την αντιβακτηριακή δράση της έναντι του βακτηρίου *P. carotovorum* subsp. *carotovorum* με τιμή ελάχιστης συγκέντρωσης αναστολής ανάπτυξης 32 mg/L.

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First record of *Hyphopichia burtonii* isolated from the storage pest *Sitophilus zeamais* and its bioactivity against mycotoxigenic fungi

M.N.P. Seco[1,2], A.K.M. Beltran[1] and M.A.O. Balendres[1,3,4]*

Summary Corn weevil (*Sitophilus zeamais)* is one of the most destructive pests of corn seeds during storage. The weevil may be a vector of mycotoxigenic fungi or yeast contaminating seed lots. In this study, an unknown yeast species was isolated from corn weevils found in stored corn seeds. We hypothesized that this yeast had an antifungal activity thereby inhibiting growth of mycotoxigenic fungi in corn seeds. The yeast species was identified as *Hyphopichia burtonii*, using combined morphological and molecular assays, and its potential inhibitory activity was assessed *in vitro* (spread plate and dual culture) against three known mycotoxigenic fungi, *Fusarium verticillioides, Aspergillus niger* and *A. flavus*. Screening of the antagonistic activity of the yeast isolate showed 50 – 69% colony growth inhibition of three fungi when the yeast was spread plated on PDA but only slight inhibition (5.8 – 13.7% growth inhibition) in the dual culture assay. The sporulation of the fungi was also affected at 57 – 96% and 29 – 40% in spread plating and dual culture assay, respectively. In addition, volatile and non-volatile fractions also showed a reduction in mycelial growth. Variable responses were observed among the mycotoxigenic fungi. Further research would be interesting on the potential utilization of the antagonistic yeast to reduce fungal growth and sporulation, and possible mitigation of mycotoxin contamination in corn grains. To our knowledge, this is the first record of *H. burtonii* isolated from an insect, specifically *S. zeamais*.

Additional keywords: corn weevil, insect-yeast interaction, seed pathology, yeast

Introduction

Corn (*Zea mays*) is one of the major staple crops grown throughout the Philippines, amounting to 2 million metric tons (Philippine Statistics Authority, 2021). About 14 million Filipinos prefer white corn as their main staple while yellow corn accounts for about 50% of livestock mixed feeds (Department of Agriculture). Much of it is produced by small-farm holders for their consumption and livelihood. Corn grits are used for human consumption, while the other parts of the seeds produced during milling may be used as animal feeds. Corn seeds are often stored prior to processing, particularly during the rainy season. Some of these seeds are also used for the next growing season.

During the postharvest and storage period, seeds are predisposed to several fungal contaminants and insect pests, which may contribute to economic loss and reduce seed quality (Balendres *et al.,* 2019). These biotic agents are estimated to cause 20% of food losses, of which up to 40-50% are from developing countries (Yun *et al*., 2018). Among the stored product pathogens and pests are *Aspergillus* species, *Fusarium* species, and *Sitophilus zeamais* (Coleoptera: Curculionidae) (corn weevil) (Ferreira-Castro *et al*., 2012).

Mycotoxins like aflatoxin, ochratoxin, and fumonisin are toxic substances produced by some fungi (mycotoxigenic fungi), namely *Aspergillus flavus, A. niger,* and *Fusarium verticillioides* (Balendres *et al*., 2019). The

[1] Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines, Los Baños, Laguna, Philippines.
[2] Department of Science and Technology-Science Education Institute, Philippines.
[3] Department of Biology, College of Science, De La Salle University, Manila, Philippines.
[4] Plant and Soil Health Research Unit, Center for Natural Sciences and Environmental Research, De La Salle University, Manila, 1004 Philippines.
* Corresponding author: mark.angelo.balendres@dlsu.edu.ph

Food and Agriculture Organization's (FAO) approximates 25% of the world's annual crop production to be contaminated with mycotoxins, leading to significant crop losses in food and feed products valued at an estimated 1 billion metric tons (Pfliegler *et al*., 2015). Meanwhile, infestation of insects in mature cobs during grain storage is common. The corn weevil, *S. zeamais,* is one of the most serious and common pests of corn in the tropics (Paes *et al*., 2012; Sori and Ayana, 2012). It has also been reported as a vector for toxigenic fungi during storage (Ferreira-Castro *et al*., 2012); thus, it may cause additional damage to stored products by spreading and promoting fungal contamination. Insect-microbiota interactions play an important role in insect biology, influencing the insect's development, physiology, nutrition, survival, immunity, or even vector competence (Malassigné *et al*., 2021).

In 2021, individuals of corn weevil were observed in corn seed samples from seed lots that usually showed high fungal contamination. When the seeds were placed in a culture medium, a yeast was found and no fungal contaminants were isolated. We hypothesized that the unknown yeast species carried by the corn weevils is bioactive against mycotoxigenic fungal seed contaminants. This study aims to 1) identify the yeast species isolated from the corn weevils using a combined conventional and DNA-based approach and 2) assess the antifungal activity of this yeast against three common mycotoxigenic fungi *F. verticillioides, A. flavus,* and *A. niger* using spread plate technique and dual culture assays.

Materials and Methods

Sample collection, yeast isolation and mycotoxigenic fungi source

Individuals of corn weevil were collected in March 2021 in Laguna, Philippines, from brown paper bags containing corn seeds. The weevils were kept in glass flasks with a mesh screen lid. For the experiment, weevils were directly plated onto Petri plates (10 individuals/plate X 3 plates) containing potato dextrose agar (PDA) medium. The plates were incubated at room temperature (28-30°C) for seven days. The yeast was isolated and purified in new PDA medium.

The three mycotoxigenic fungi used in this study, *A. flavus, A. niger,* and *F. verticillioides,* were obtained from the fungal repository of the Plant Pathology Laboratory, Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños. These fungi were previously isolated from stored corn seeds and were already identified using molecular assays. The fungi were also found to contain the aflatoxin, ochratoxin, and fumonisin biosynthesis genes and metabolites, determined using a polymerase chain reaction (PCR) assay and ELISA kit, respectively.

Yeast identification

Identification of the yeast species was done through a combined morpho-cultural and molecular assays. Morphocultural characterization was done in yeast extract peptone glucose agar (YEPG) (10 g yeast extract, 20 g peptone, 10 g glucose, 15 g agar). The insect genomic DNA was extracted using the CTAB method (Doyle and Doyle, 1987). The sequences of the D1/D2 domain using the LR0R (ACCCGCTGAACTTAAGC) and LR5 (TCCTGAGGGAAACTTCG) (Vilgalys, 1988) primer pairs were used to amplify the LSU rRNA gene region of the yeast in a PCR assay. The 25 µL PCR mix contained 1x PCR buffer (Invitrogen), two mM MgCl2 (Invitrogen), 0.2 mM dNTP mix (Invitrogen), 0.2 µM of each primer, 1 U of *Taq* polymerase (Invitrogen), one µL DNA template and DEPC-treated water (Invitrogen). PCR reactions were performed using MyCycler™ Thermal Cycler System (Bio-Rad Laboratories) with an initial denaturation for 5 min at 95°C, 35 cycles of 30-sec denaturation at 95°C, 30-sec annealing at 55°C, and 1 min extension at 72°C, followed by a final extension for 10 min at 72°C for LSU rRNA gene and an initial denaturation for 5 min at 94°C, 35 cycles of 30-sec denaturation at 94°C, 30-sec annealing at 55°C, and 30-sec extension at 72°C.

The amplified products were visualized using the Molecular Imager GelDoc™ XR+ with Image Lab software (Bio-Rad Laboratories), and amplified PCR products were sent to 1st Base (Malaysia) for DNA sequencing.

Weevil Identification

A PCR assay, using the same conditions as mentioned above, was also performed to validate the identity of the corn weevil. Primer pairs LCO1490 (GGTCAACAAATCATAAAGATATTGG) and HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer *et al*., 1994) of the COI gene were used in the PCR assay.

DNA Sequence Analysis

Sequence assembly was performed using the Geneious program. The obtained consensus sequences were compared pairwise using a BLASTN search (NCBI GenBank). Sequences were then aligned with the sequences of related species retrieved from GenBank using multiple alignments. A phylogenetic tree was constructed using the maximum likelihood method of MEGA X software, and confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates).

***In vitro* screening of yeast antagonistic assay**

Hyphopichia burtonii was tested on each fungal isolate to determine the yeast's effect on fungal growth and spore production in a spread plate technique following the method of Souza *et al.* (2017). The yeast was grown in culture media and a concentration of 10^7 cells mL^{-1} was obtained. For the fungal isolates, suspension of spores was obtained at 10^6 spores mL^{-1}. Aliquots of 100 µL of the yeast suspension were spread using a Drigalsky handle on Petri dishes containing PDA medium. Then, aliquots of 10 µL of each fungal spore suspension were placed in the center of the Petri dishes. This was performed in triplicates and the Petri dishes were incubated at 28°C for seven days. Positive control for the growth of each fungal isolate was conducted by inoculating the spores without inoculating the yeast isolates. The colony diameter of each fungus was measured to evaluate the mycelial vegetative growth, and the percentage of growth inhibition was obtained, considering that the positive control was 100% of the diameter. The same treatments for growth inhibition were conducted to analyze spore production. After seven days, the spores were counted by placing three agar blocks per replicate in 10 ml of sterile distilled water. The percentage of inhibition of spore production was obtained considering the spore concentration in the positive control as 100% for each fungal isolate.

A dual culture assay (Moradi *et al*., 2020) was also performed by placing 50 µl of an actively growing suspension of *H. burtonii* (10^8 cells mL^{-1}) and streaking 3.5 cm away from the center of the YEPG plates containing 10 g yeast extract, 20 g peptone, 10 g glucose, and 15 agar and subsequently incubated at 25°C for 24 h. Post-incubation, 10 µL each of the three mycotoxigenic fungal spore suspensions (10^6 spores mL^{-1}) was placed in the center of each Petri dish and incubated at 28°C in the dark. The inhibition of radial fungal growth was recorded for up to 7 days. Fungal growth with no yeast inoculum was used as a control. The ability of strains to inhibit fungal growth was calculated with the following equation: $I = C\text{-}T/C \times 100$, where I is the inhibition of mycelial growth (%), C is the growth of the fungal pathogen in control Petri dishes, and T is the growth in the interaction assays. The percentage of inhibition of spore production was also conducted.

Effect of heating (volatile and non-volatile compounds) on the antimicrobial activity of *H. burtonii*

To assess the effects of volatile compounds on the mycelial growth of fungi, the *H. burtonii* strain was streaked out on YEPG plates and incubated for two days at 28°C (Moradi *et al.,* 2020). Ten µL each of the three mycotoxigenic fungal spore suspensions (10^6 spores mL^{-1}) of 7-day-old fungi were placed in the center of the plate and upside down on a Petri dish containing the 48-hour inoculated *H. burtonii* strain. The plates were

then sealed with paraffin film (Parafilm, Sigma-Aldrich, Germany) and incubated for seven days at 27°C in the dark. The fungi's colony diameter (mm) was measured after seven days. The YEPG plates with no yeast isolate applied were used as a control.

In the non-volatile compound assay, *H. burtonii* strain was cultured in individual flasks containing 75 ml of sterilized potato-dextrose broth (PDB). It was placed on a rotary shaker (150 rpm) at room temperature to promote the growth of yeast strain. After four days, the suspensions were passed through No. 1 filter paper (Whatman, Sigma-Aldrich, Germany) and autoclaved. The sterilized suspension was mixed with YEPG at three ratios (5%, 15%, and 25%) and poured into Petri dishes. Fungal suspensions were inoculated on the center of the plates at 10 µL (10^6 spores mL^{-1}). The mycelial growth of the fungi was monitored and recorded at seven days.

Statistical Analysis

A paired sample (Independent) T-test and ANOVA test were performed using Statistical Tool for Agricultural Research (STAR Nebula) with a 95% confidence level.

Results

Yeast incidence and identity

The yeast grew from all corn weevils (100% incidence) plated in the PDA medium after seven days (Fig. 1A). No other microbes were observed growing from the corn weevil samples. Morphocultural characterization showed white, powdery, flat, filamentous colonies with yeast-like cells which are ellipsoidal or pyriform in shape at about 2.5-5.0 x 1.5-2.5 µm with thin, smooth walls with septate hyphae that are 4-6 µm broad, and dichotomously branched (Fig. 1B and 1C). Analysis of the D1/D2 domain of the LSU rRNA gene amplified a PCR product of 860 bp (Fig. 2B) and NCBI BLAST results showed 100% similarity to *Hyphopichia burtonii* (CP024760) (Table 1). The phylogenetic tree was generated based on the sequences of the D1/D2 domain of the LSU rRNA gene also revealed that the strain formed a cluster with known *H. burtonii* (Fig. 3).

Weevil identity

PCR product of the weevil's DNA analyzed using COI gene showed 700 bp at 1.5% agarose gel electrophoresis (Fig. 2A). The DNA sequences of the COI gene of the individuals revealed 99.14% similarity to *Sitophilus zeamais* (NC030764) (Table 2).

Yeast's antifungal activity

Four *in vitro* assays were conducted to test antifungal activity of *H. burtonii* against three mycotoxigenic fungi. In the spread plate assay, *H. burtonii* significantly inhibited the colony growth of three fungi with a 50-69% reduction on spread plate assay (Fig.

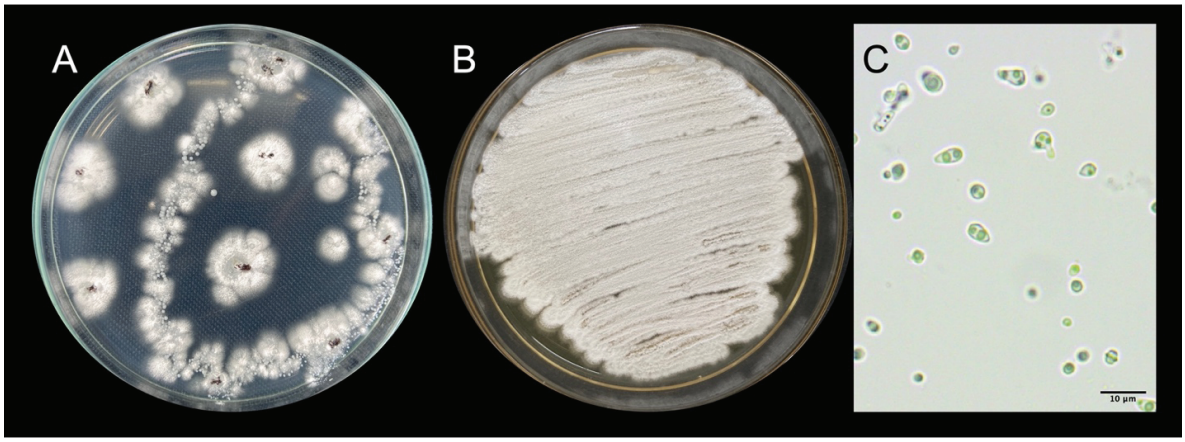

Figure 1. Isolation of mycoflora in corn weevil by direct plating on PDA: (A) pure culture of *Hyphopichia burtonii* grown in yeast extract peptone glucose agar (YEPG) at 3 days incubation (B) and yeast cells at 1000x magnification (bar=10 µm) (C).

4A and Fig. 5). Spore production was also inhibited at 57-96% where highest inhibition was recorded with *A. flavus* at 96%, followed by *A. niger* at 87.85% and *F. verticillioides* at 57.63% (Table 3). In the dual culture assay, the isolate significantly inhibited the growth of *F. verticillioides* and *A. niger* at 13.68% and 6.31% respectively. However, no significant inhibition was observed on *A. flavus* (Fig. 4B and Fig. 6). On fungal spore production, all fungi were significantly inhibited (29-40%) (Table 4). *H. burtonii* inhibited the mycelial growth of the three fungi which is correlated with the inhibition of spore production.

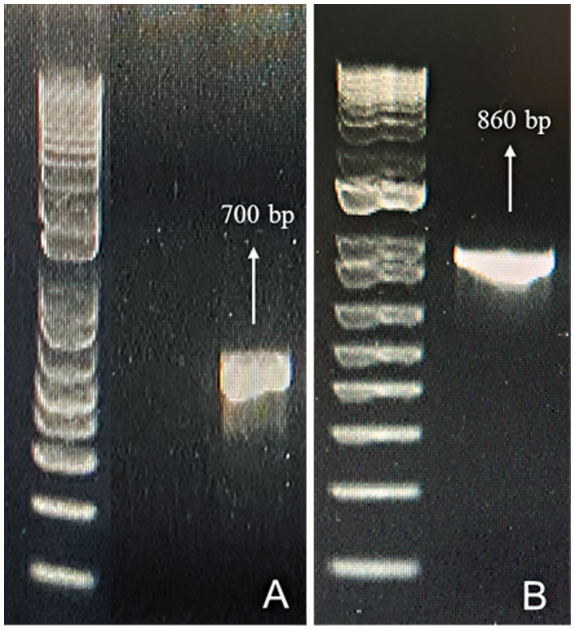

Figure 2. Agarose gel electrophoresis (1.5%) of PCR product of *Sitophilus zeamais* at 700 bp using COI gene (A) and *Hyphopichia burtonii* at 860 bp using LSU rRNA gene (B).

The test for the presence of volatile compounds of *H. burtonii* also showed a reduction in mycelial growth in *F. verticillioides* and *A. niger* with 17.41 and 58.89% inhibition, respectively, while *A. flavus* growth was not significantly affected (Fig. 7A). For the non-volatile compound test, higher yeast concentration amended in YEPG (15% and 25%) was efficient in inhibiting the growth of *F. verticillioides* (25-30% inhibition) and *A. flavus* (7.41-14.44%) but did not affect *A. niger* growth (Fig. 7B). At 5% yeast concentration, no inhibition was observed in *F. verticillioides* and *A. flavus*. However, significant

Table 1. Sequences retrieved from GenBank according to the closest BLAST results of the D1/D2 domain of the LSU rRNA gene.

Isolate	Closest sequence match	Source/Host	Country	Coverage	Similarity
MB Corn Weevil Yeast Isolate 001	CP024760.1 *Hyphopichia burtonii*	nuruk	South Korea	100	100
	KY107882.1 *Hyphopichia burtonii*	inse	NI	100	100
	HF952839.2 *Hyphopichia burtonii*	Farm silage	Netherlands	100	100
	MH867400.1 *Hyphopichia burtonii*	NI	Netherlands	99	100
	NG_054819.1 *Hyphopichia burtonii*	NI	NI	96	100
	CP024761.1 *Hyphopichia burtonii*	nuruk	South Korea	88	100
	KY106445.1 *Hyphopichia fennica*	NI	Finland	95	97.09
	KY107885.1 *Hyphopichia burtonii*	Homo sapiens	NI	82	100
	JQ733412.1 *Pichia* sp.	Halophila ovalis	China	93	96.15
	CP024757.1 *Hyphopichia pseudoburtonii*	nuruk	South Korea	100	90.41

Abbreviations: (NI) No information.

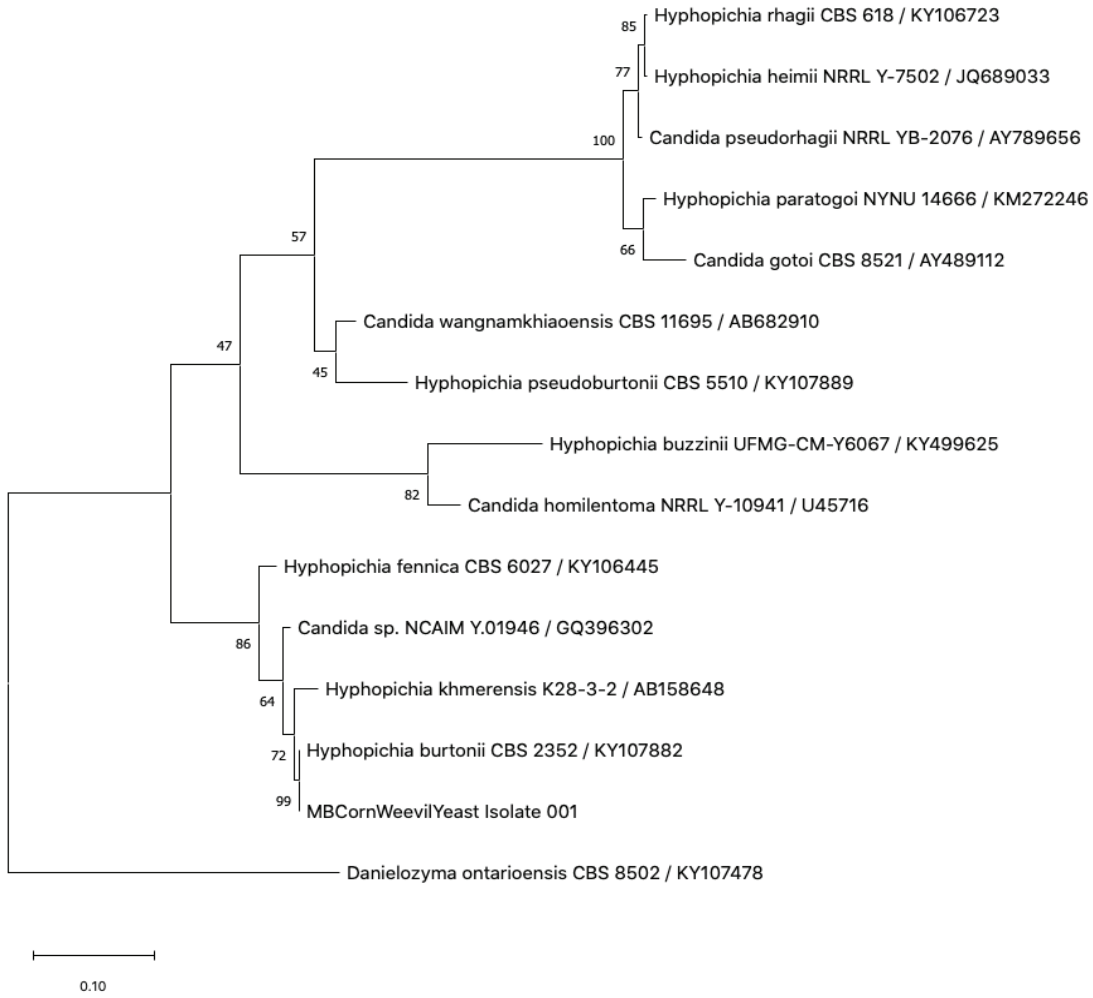

Figure 3. Phylogenetic tree based on the sequences of the D1/D2 domain of the LSU rRNA gene, showing positions of *Hyphopichia burtonii* with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data using maximum likelihood method. The numbers at nodes indicate the percentages of bootstrap sampling, derived from 1,000 samples. *Danielozyma ontarioensis* was the outgroup species in the analysis.

inhibition was observed in *A. niger* at the lowest yeast concentration.

Discussion

The corn weevil, *S. zeamais*, is known to be a vector of fungi, some of which produce toxins (aflatoxin and fumonisin). In this study, a yeast was isolated from individuals of corn weevil using combined morpho-cultural and molecular assays, and was identified as *H. burtonii*. To our knowledge, this is the first scientific report of *H. burtonii* isolated from a storage-product insect pest. As there were no fungi growing on weevils where the yeast was isolated, it was hypothesized that the yeast inhibited the activity of mycotoxigenic fungi. Yeast has been regarded as potential biocontrol agent against toxigenic fungi in stored grains (Petersson and Schnürer, 1998; Petersson *et al*., 1998; Masoud and Kaltoft, 2006). In the current study, we confirm that the *H. burtonii* isolated from corn weevil had inhibitory activity on the growth and spore production of three mycotogenic fungi, namely *A. flavus, A. niger,* and *F. verticillioides*.

The morphology and colony features of the *H. burtonii* isolated strain in this study corroborate with the description of Arx and

Table 2. Sequences retrieved from GenBank according to the closest BLAST results of cytochrome oxidase subunit I (COI) gene.

Isolate	Closest sequence match	Source/Host	Country	Coverage	Similarity
MBCornWeevil	NC_030764.1 *Sitophilus zeamais*	NI	NI	99	99.14
	AY131100.1 *Sitophilus zeamais*	NI	NI	99	99.14
	MT294139.1 *Sitophilus zeamais*	Sorghum grains	China	99	98.99
	MN905575.1 *Sitophilus zeamais*	NI	China	99	98.99
	KU757289.1 *Sitophilus zeamais*	NI	China	99	98.99
	MK649856.1 *Sitophilus zeamais*	NI	NI	94	100
	OQ533509.1 *Sitophilus zeamais*	NI	Pakistan	94	99.85
	KY912951.1 *Sitophilus zeamais*	NI	China	94	99.85
	KY912950.1 *Sitophilus zeamais*	NI	China	94	99.85
	KM459446.1 *Sitophilus zeamais*	NI	India	94	99.85

Abbreviations: (NI) No information.

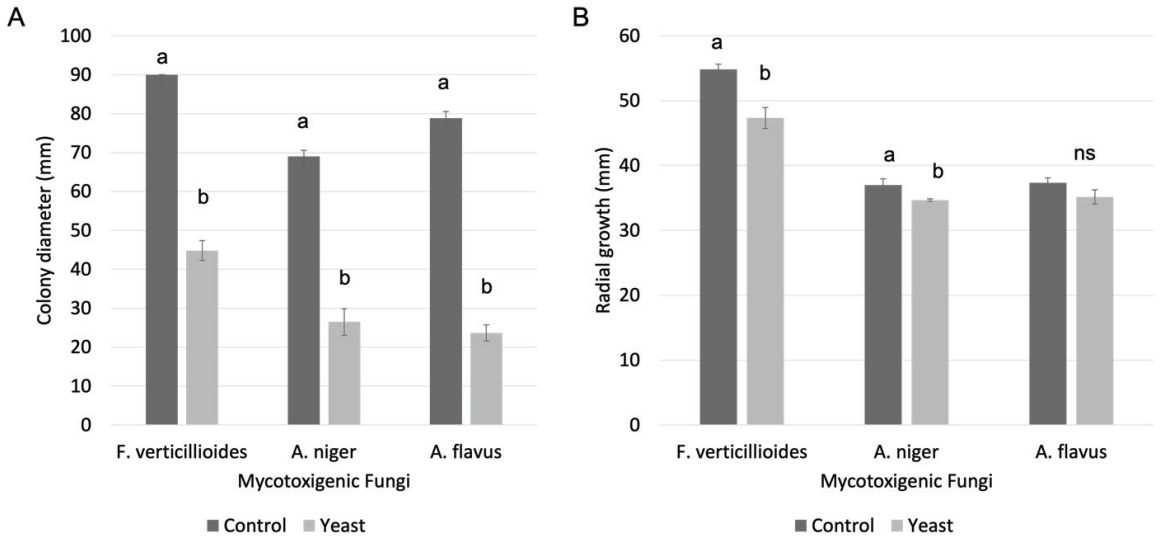

Figure 4. Colony growth of three mycotoxigenic fungi using spread plate (A) and dual culture test (B) of *Hyphopichia burtonii* at 7 days incubation. Bars, within a figure, with different letter indicate means were significant at $p<0.05$ based on T-test analysis. Values represent the mean of the two trials performed.

Vab Der Walt (1976) and Pitt and Hocking (2009). This species was formerly known as *Pichia burtonii*, a wide-spread yeast causing food and beverage spoilage. It is also called «chalk molds» because it causes defects on partially baked bakery products, cured

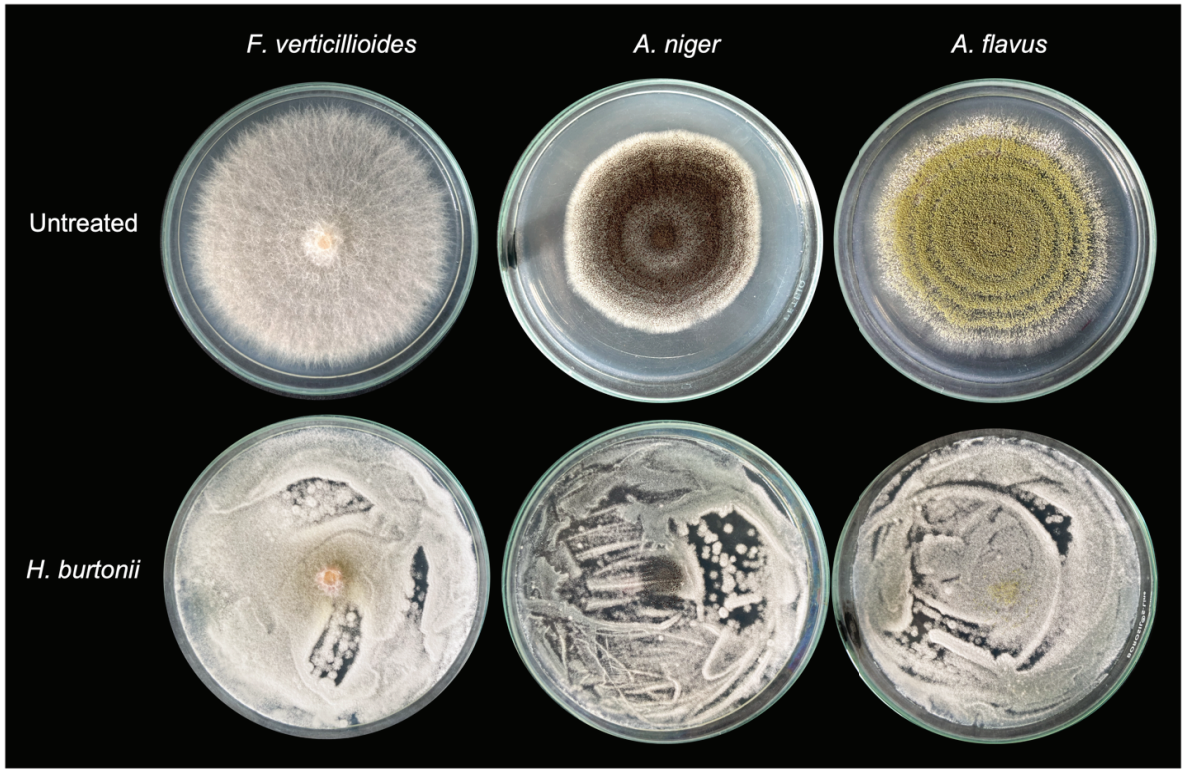

Figure 5. Spread plate assay of the antagonistic activity of *Hyphopichia burtonii* against three mycotoxigenic fungi: *Fusarium verticillioides, Aspergillus niger, Aspergillus flavus* (L-R).

Table 3. Colony growth and spore inhibition of three mycotoxigenic fungi to *Hyphopichia burtonii* strain using spread plate assay.

Treatment	Colony diameter (mm)			Spores/ml		
	F. verticillioides	*A. niger*	*A. flavus*	*F. verticillioides*	*A. niger*	*A. flavus*
Control	90.00a	69.00a	78.83a	$1.97x10^5$a	$4.06x10^6$a	$6.63x10^6$a
H. burtonii	44.83b	26.50b	23.67b	$8.33x10^4$b	$4.93x10^5$b	$2.65x10^5$b
Inhibition(%)	50.19	61.59	69.98	57.63	87.85	96.00

meat, and cookies (Lee and Fujio, 1999; Simoncini *et al*., 2007). The species has been reported in a wide variety of substrates, particularly high starch substrates such as corn, wheat, and rice, and from insects and water from fish ponds (Kurtzman, 2011). *Hyphopichia burtonii* has been isolated from grass insects in Thailand (Limtong *et al*., 2012), coffee berry borer (*Hypothenemus hampei*) from a coffee tree in Brazil (Moreira, 2012), wild edible crickets (*Gryllus bimaculatus)* in Kenya (Gatheru, 2019), red flour beetle (*Tribolium castaneum)* in rice in Korea (Yun *et al*., 2018), guts of insect larvae and decayed woods from Henan Province, Central China (Ren *et al*., 2015), and insect frasses (Kurtzman, 2011). In addition, *H. burtonii* has also been reported in rotting woods, the leaf surface of a mango tree, and a freshwater lake in Brazil (Ribeiro *et al*., 2017), spoiled foodstuffs, caterpillars, silage, pollen, fish feeds, and fishponds (Pinheiro *et al*., 2018; Barnett *et al*., 2000). The current study, to our knowledge, is the first scientific report of *H. burtonii* isolate from corn weevil (*S. zeamais*).

Moreira (2012) reported that *H. burtonii*

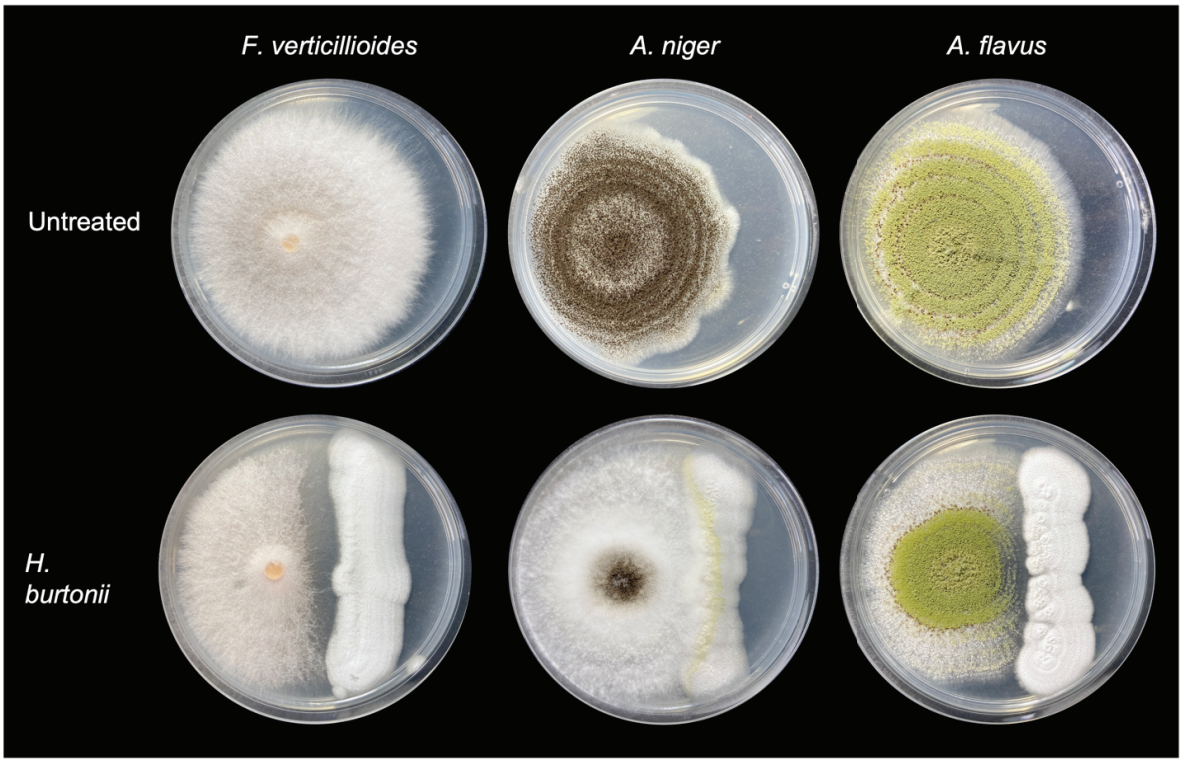

Figure 6. Dual culture assay of the antagonistic activity of *Hyphopichia burtonii* against three mycotoxigenic fungi: *Fusarium verticillioides, Aspergillus niger, Aspergillus flavus* (L-R).

Table 4. Colony growth and spore inhibition of three mycotoxigenic fungi to *Hyphopichia burtonii* strain using dual culture assay.

Treatment	Radial growth (mm)			Spores/ml		
	F. verticillioides	*A. niger*	*A. flavus*	*F. verticillioides*	*A. niger*	*A. flavus*
Control	54.83a	37.00a	37.33ns	1.7 x10^5a	6.2 x10^6a	4.2 x10^6a
H. burtonii	47.33b	34.67b	35.17	1.0 x10^5b	3.5 x10^6b	3.0 x10^6a
Inhibition(%)	13.68	6.31	5.80	40.00	43.39	28.58

parasitizes the coffee berry borer *Hypothenemus hampei* (Coleoptera: Scolytidae). In the current study, we did not observe *H. burtonii* parasitizing the corn weevil. Nevertheless, further research is necessary to determine the associations of *H. burtonii* with the corn weevil, aside from the latter being a vector of the yeast. *Hyphopichia burtonii* has also been associated with cutaneous infection in Barbastelle bats (Simpson *et al*., 2013). In 2021, a strain of *H. burtonii* was reported, for the first time, to be associated with peritonitis in humans on peritoneal dialysis, which may require specific media and other detection techniques (Chamroensakchai *et al.,* 2021). The yeast strain associated with the corn weevil in our study was easily cultured in PDA medium within seven days. Further research is needed to compare the biology and pathology of *H. burtonii* isolates from various sources as these may differ based on their host (pathogenic or symbiotic) and host specificity is also possible. A genomics approach may also shed light as to whether the *H. burtonii* isolates from various substrates and samples (plants, insects, and

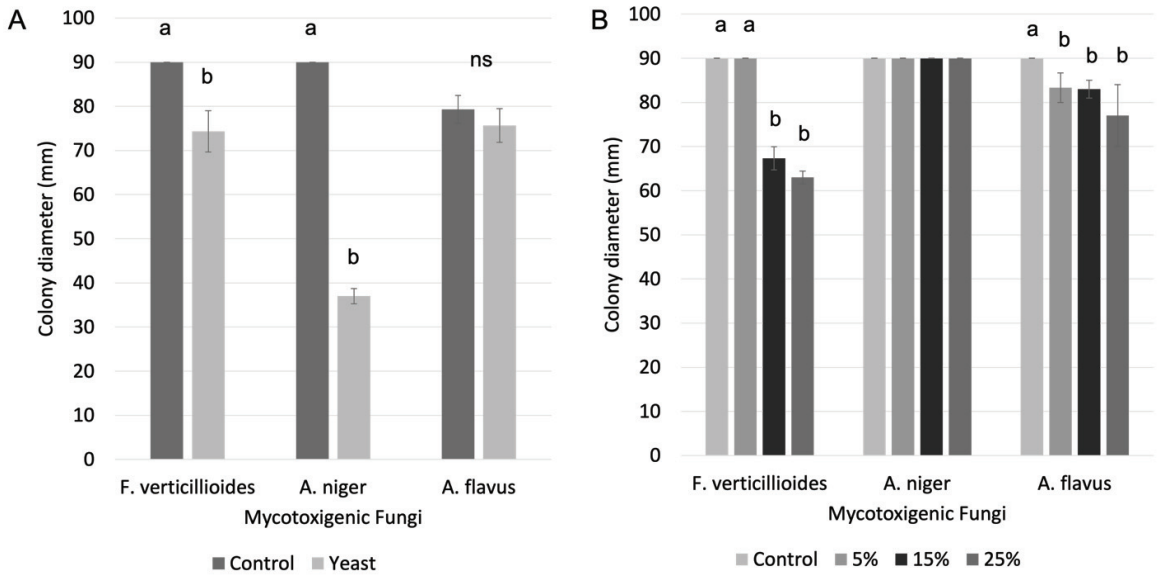

Figure 7. Colony diameter (mm) of three mycotoxigenic fungi on test for volatile (A) and non-volatile compounds (B) of *Hyphopichia burtonii* at 7 days incubation. Bars, within a figure, with different letter indicate means were significant at $p<0.05$ based on T-test analysis and ANOVA test.

animals) are the same or different strains. Care must be considered when working on stored grains and other products (bread, beverages, and other substrates) where *H. burtonii* has been previously reported.

The role of *H. burtonii* in insects and other hosts is not yet fully understood. Studies have indicated that it might play important roles in their hosts by detoxification of food materials and help on the supply of essential nutrients (Ren *et al*., 2015) or parasitizing insects and other hosts (Moreira, 2012; Simpson *et al*., 2013; Chamroensakchai *et al*., 2021). In the present study, we showed the antifungal activity of *H. burtonii* isolated from corn weevil to the growth and spore production of three mycotoxigenic fungi *in vitro*. The results showed that the method of strain inoculation (yeasts and fungi) differed in the level of its inhibition effect on mycelial growth and spore production. *Hyphopichia burtonii* highly-suppressed the mycelial growth and spore production of the three fungi when spreading the yeast isolate in the agar prior to the inoculation of the fungi. The results corroborate with those of Souza *et al*. (2010), where the production of toxigenic fungal spores was inhibited by 100% by wild yeasts.

In contrast, dual culture significantly inhibited mycelial growth and spore production of the three fungi but at a lower rate compared with the spread-plated yeast. A difference in the degree of inhibition by the yeast isolate was also observed among the three mycotoxigenic fungi: the lowest inhibition was obtained against *A. flavus*, followed by *A. niger*, then *F. verticillioides*. In the case of *A. flavus,* the yeast inhibited sporulation but did not interfere with mycelial growth. Unlike the spread plate assay, the dual test allowed the growth of the yeast and the fungi on each side, thus, giving time for the fungi to grow before interacting with the yeast isolate. It could be that the *Aspergillus* species spreads faster than *F. verticillioides* and explains the low inhibition effects on these two species. Similarly, Ramos *et al*. (2010) explained yeast isolates' more significant inhibitory effect on sporulation than on mycelial development when they inoculated the yeast isolates concomitantly with the inoculation of filamentous *Aspergillus* fungi, namely *A. carbonarius* and *A. ochraceus*, 4 cm apart, allowing both isolates to develop before the direct contact of the yeast colony with the fungi. Moreover, Ramos *et al*. (2010) and Souza *et al*. (2017) re-

ported that the concentration of the yeast inoculum and the degree of inhibition were directly proportional: the higher the inoculum concentration of the yeast species, the greater the inhibitory effect on sporulation.

The volatile and non-volatile tests in *H. burtonii,* indicate that the yeast may contain volatile and non-volatile compounds with different effect on the fungi at different yeast concentrations since the volatile compounds significantly inhibited *A. niger,* while non-volatile compounds at concentration 15% inhibited mycelial growth of *F. verticillioides* (25% inhibition). and at concentration 5%, of *A. flavus* (7% inhibition).

Overall, the different inhibition effect of *H. burtonii* on the three mycotoxigenic fungi mycelium growth and spore production in dual culture, volatile compound, and non-volatile compound tests, can be related to different mechanisms of competitive interactions between the yeast strain and the fungi. A high yeast population may likely restrict the availability of nutrients and sites for colonization, which are essential for the germination of spores (Björnberg and Schnürer, 1993). Antagonistic yeasts possess several mechanisms of action, including competition for nutrients and space, production of cell wall degrading enzymes, volatile organic compounds, non-volatile compounds, and direct mycoparasitism (Freimoser *et al*., 2019). Volatile compounds are known to act directly against the pathogens (direct antibiosis) by destroying the cell wall or indirectly inducing systemic resistance to the plant (Chen *et al*., 2008; Zheng *et al*., 2013). Identification of these compounds may lead to the their use as chemical agents against mycotoxigenic fungi.

Conclusion

The yeast *H. burtonii* which was isolated from the storage pest corn weevil (*S. zeamais*) for the first time was antagonistic to three mycotoxigenic fungi (*A. flavus, A. niger,* and *F. verticillioides)* of corn by inhibiting fungal growth and spore production. Thus, the research contributes with knowledge for the potential utilization of *H. burtonii* against mycotoxigenic fungi, and the reduction of mycotoxins in stored products.

Declarations

Author contributions

Conceptualization, design and supervision, MAB and AKB; conduction of experiments, design and generation of figures , analysis of data and writing—original draft preparation, MNS; interpretation of results, MNS and MAB; writing—review, editing and final version of the manuscript, MAB and AKB. All authors have read and agreed to the published version of the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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Πρώτη καταγραφή του *Hyphopichia burtonii* που απομονώθηκε από το έντομο αποθηκών *Sitophilus zeamais* και η βιοδραστηριότητά του έναντι μυκοτοξινογόνων μυκήτων

M.N.P. Seco, A.K.M. Beltran και M.A.O. Balendres

Περίληψη Ο βρούχος του καλαμποκιού (*Sitophilus zeamais*) είναι ένα από τα πιο καταστροφικά έντομα των σπόρων του αραβοσίτου κατά την αποθήκευση. Το έντομο δύναται να είναι φορέας μυκοτοξινογόνων μυκήτων ή ζυμομυκήτων που επιμολύνουν τους σπόρους. Σε αυτή τη μελέτη, ένα άγνωστο είδος ζυμομύκητα απομονώθηκε από άτομα του *S. zeamais* που εντοπίστηκαν σε αποθηκευμένους σπόρους αραβοσίτου. Υποθέσαμε ότι ο συγκεκριμένος ζυμομύκητας είχε αντιμυκητιακή δράση αναστέλλοντας την ανάπτυξη μυκοτοξινογόνων μυκήτων στους σπόρους του αραβοσίτου. Το είδος του ζυμομύκητα ταυτοποιήθηκε ως *Hyphopichia burtonii*, με τη συνδυασμένη εφαρμογή μορφολογικών και μοριακών μεθόδων, και αξιολογήθηκε *in vitro* (επίστρωση σε τριβλίο PDA και διπλή καλλιέργεια) η πιθανή ανασταλτική δράση του στην ανάπτυξη τριών γνωστών μυκοτοξινογόνων μυκήτων,

Fusarium verticillioides, Aspergillus niger και *A. flavus*. Ο έλεγχος της ανταγωνιστικής δράσης της απομόνωσης του ζυμομύκητα έδειξε 50 – 69% και 5,8-13,7% αναστολή ανάπτυξης της αποικίας των μυκήτων, όταν χρησιμοποιήθηκαν οι μέθοδοι της επίστρωσης σε τριβλίο και της διπλής καλλιέργειας, αντίστοιχα. Επίσης επηρεάστηκε η παραγωγή σπορίων από τους τρεις μύκητες σε ποσοστό 57 – 96% και 29 – 40%, στη μέθοδο της επίστρωσης σε τριβλίο και της διπλής καλλιέργειας, αντίστοιχα. Επιπλέον, τα πτητικά και τα μη πτητικά κλάσματα προκάλεσαν μείωση στην ανάπτυξη των μυκηλίων. Παρατηρήθηκαν διαφορετικές αποκρίσεις μεταξύ των μυκοτοξινογόνων μυκήτων. Περαιτέρω έρευνα θα είχε ενδιαφέρον για την πιθανή χρήση του ανταγωνιστικού ζυμομύκητα για τη μείωση της ανάπτυξης του μυκηλίου και της σποριοποίησης των μυκοτοξινογόνων μυκήτων και τον πιθανό μετριασμό της επιμόλυνσης σπόρων αραβοσίτου με μυκοτοξίνες. Από όσο γνωρίζουμε, αυτή είναι η πρώτη καταγραφή απομόνωσης του ζυμομύκητα *H. burtonii* από έντομο και ειδικότερα από το *S. zeamais*.

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Population monitoring and status evaluation of the new invasive pest, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), in various crop systems of Georgia (Sakartvelo)

G. Japoshvili[1], M. Murvanidze[2]*, N. Inasaridze[2], N. Meskhi[3], Z. Lipartia[3] and L. Namicheishvili[4]

Summary The spotted wing drosophila, *Drosophila suzukii* (Matsumura, 1931), population fluctuations and distribution were monitored in four susceptible crops (blueberry, strawberry, cherry, wine grapes) and five major agricultural regions of Georgia (Guria, Samegrelo, Imereti, Kartli and Kakheti) during the years 2021 and 2022 with the aim to study the population size and evaluate the pest status. Population monitoring was conducted in four locations of each studied region on a weekly basis from May to November using PHEROCON® SWD traps with PHEROCON® SWD PEEL-PAK™ Broad Spectrum Lures. The investigation indicated significant growth of population from 2021 to 2022 in most of the studied locations. Population increase was detected in all crop orchards except cherries. The absence of alternative host plants at crop proximity was of critical importance to save the crop from pest invasion. The crop plant species did not have a significant impact on *D. suzukii*. Pest population was significantly larger in summer and autumn compared to spring, possibly influenced by the ripening of alternative crops such as blackberry and elderberry. The sex ratio between male and female individuals was almost 1:1 and remained consistent through the two-year study period and across regions. We consider that *D. suzukii* entered the country from the southwestern part and extended its distribution range towards the east. No strategies for *D. suzukii* control have been elaborated in Georgia so far. Sprays of effective pesticides based on pest monitoring as well as sanitation measures involving removal of alternative host plants and any crop residues from the field are necessary to avoid pest outbreak.

Additional keywords: Georgia, pheromone traps, population monitoring, spotted wing drosophila

Introduction

The spotted wing drosophila (SWD), *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae), has become a major invasive pest impacting small fruits such as berries and stone fruits in America and Europe. The pest is native to Asian countries (Kanzawa, 1939; Peng, 1937; Lee *et al*., 2011; Walsh *et al*., 2011; Asplen *et al*., 2015). In North America it was first detected in Hawaii in 1980 and on the mainland (California) in 2008 (Lee *et al*., 2011; Walsh *et al*., 2011). In Europe (Italy and Spain) SWD was detected in 2009 (Calabria *et al*., 2012) and currently it is distributed across the whole Western Europe (Asplen *et al*., 2015) with a continuous expansion of its range. While Caucasian ecoregion is located on the favored latitude for the pest (40 – 47°) (Calabria *et al*., 2012), the presence of SWD was officially registered in 2017, only when it was accidentally discovered in traps installed close to the agricultural markets in Khelvachauri municipality and Batumi city (Western Georgia) (Japoshvili *et al*., 2018).

Although SWD prefers small and soft fruits like cherries and berries, its host range includes apricots, nectarines, figs, grapes and peaches (Stacconi, 2022). The total yield loss by damage of SWD reached up to 80% of strawberry crops in France, and 30-40% of various berries in Italy in 2010. Economic losses also include labor fees, costs for

[1] Institute of Entomology. Agricultural University of Georgia. 240, D. Aghmashenebeli Alley, 0131 Tbilisi. Georgia.
[2] Faculty of Exact and Natural Sciences. I. Javakhishvili Tbilisi State University. 1, I. Chavchavadze ave. 0179 Tbilisi. Georgia.
[3] Department for Plant Protection. National Food Agency. 6, Marshal A. Gelovani ave. 0159 Tbilisi, Georgia.
[4] CNFA's representative office in Georgia. 47 Kostava street, Tbilisi 0179, Georgia.
* Corresponding author: maka.murvanidze@tsu.ge

chemicals, and other expenses (Lee *et al*., 2011).

Georgia is considered as one of the promising contributors of blueberry export in Eastern Europe with increasing share in country's export market. In 2019, the country's income from blueberry export equaled to 983.3 thousand US$, while according to the 2023 data from January to July, the income has increased to the 21,228.14 thousand US$ (https://www.geostat.ge/). On the other hand, Georgia is an ancient wine-producing country (McGovern *et al*., 2017), and wine is considered as one of the major export products, accounting for 4.2% share of the country's overall export in 2022. Vineyards occupy 41.2% of the country's cropland territory (https://www.geostat.ge/).

The growing demand for agricultural products encourages Georgian farmers to expand cultivation areas, thus increasing the preferable host range for SWD. Although a population outbreak has not been recorded, monitoring and evaluation of the population size of SWD in Georgian susceptible croplands has not yet conducted. This is the first attempt to monitor and evaluate population of *D. suzukii* across the whole country in different susceptible crops such as blueberry, strawberry, cherry and grape. The goal of this paper is to: 1) monitor the population of *D. suzukii* in susceptible crop orchards located in Guria, Samegrelo, Imereti, Kartli and Kakheti regions of Georgia for two consecutive years; 2) evaluate the pest status with the aim elaborating effective management tools to prevent a population outbreak from reaching economically damaging levels.

Material and Methods

Population monitoring of *D. suzukii* was conducted in major agricultural regions in both western and eastern parts of Georgia (Guria, Samegrelo, Imereti, Kartli and Kakheti) during the years 2021 and 2022, from May (5th and 15th, respectively) to October. The major susceptible agricultural crops which are commercially produced countrywide, were included: blueberries, strawberries, wine grapes and cherries.

The regions Guria and Samegrelo are located in Plain and Piedmont hilly subtropical humid (Kolkhic forest) landscapes with subtropical climate, influenced by close vicinity of Black Sea, with high annual rainfall (1400-1500mm) and annual average temperature 14°-15°C. In Imereti, the impact of Black Sea is less remarkable, the annual precipitation is 800-1300mm and the annual average temperature is 7°-14°C. The Kartli region is characterized by Plain and Piedmont hilly sub-Mediterranean semi-humid landscapes with forest and shibliak landscapes, 400-500mm annual precipitation and 10°-13°C annual average temperature. Moderately warm plain semi-humid forest landscapes transient to the subtropical type are spread within the limits of Alazani Plain of Kakheti region, where our investigation was conducted, with 400-600mm annual precipitation and 11°-13°C annual average temperature (Bolashvili and Neidze, 2022; Elizbarashvili, 2007).

In Guria investigations were carried out in three blueberry and one strawberry field, in Samegrelo in three blueberry fields, in Imereti in three vineyards and one strawberry field, in Kartli in three cherry orchards and one strawberry field and in Kakheti in three vineyards and one strawberry field. In each region, four cropland sites of 2ha area were selected, with three sites representing the major susceptible crops for SWD (blueberry, vine grape or cherry) and one strawberry field. (Table 1). The distances between the sites within the regions ranged from 3 to 5 km (Table 1, Fig. 1). Each site received the scheduled pesticide applications by the farmers according to the schemes provided by the National Food Agency (www.nfa.gov.ge).

For the SWD population monitoring, PHEROCON® SWD Trap with PHEROCON® SWD PEEL-PAK™ Broad Spectrum Lures (Trécé Inc., Adair, OK, USA) were used. In 2021, the traps were installed in the first week of May, while in 2022, they were placed in the last week of April. Five traps were placed

Table 1. List of in five major agricultural regions of Georgia (Guria, Samegrelo, Imereti, Kartli and Kakheti) and monitoring locations/sites (2ha size of each) of *Drosophila suzukii* with GPS coordinates and key crops where monitoring traps were located.

Region	Monitoring Location	Number of traps	GPS coordinates and altitude	Key crop and dominant cultivar	Site description
Guria	Laituri	5	N41.917912° E41.867443° 25m a.s.l	Blueberry: Legacy	Homogeneous blueberry plantation, scarce wild vegetation in surroundings. Drip irrigation. Pesticide application scheme proposed by nfa.gov.ge. Imported bumble bees (*Bombus terrestris*) for pollination
	Naruja	5	N41.904938° E41.958540° 135m a.s.l	Blueberry: Legacy	Homogeneous blueberry plantation, wild vegetation by herbs including elderberry. Drip irrigation. IPM scheme proposed by nfa.gov.ge.
	Natanebi	5	N41.910132° E41.787358° 0m a.s.l.	Strawberry: Fortuna	Strawberry plantation with dense wild vegetation around, mostly elderberry Drip irrigation. Pesticide application scheme proposed by nfa.gov.ge.
	Tsetskhlauri	5	N41.871870° E41.873768° 57m a.s.l.	Blueberry: Legacy	Homogeneous blueberry plantation, scarce wild vegetation in surroundings. Drip irrigation. IPM scheme proposed by nfa.gov.ge.
Imereti	Melauri	5	N42.193969° E42.367201° 33m a.s.l.	Strawberry: Elsanta	Subsistence farm. Multicropping. Different crop varieties harvested at different periods of season. No insecticide application. Fungicides and NPK fertilizers applied
	Obcha	5	N42.123688° E42.89659° 181m a.s.l.	Grapevine: Tsitska and Tsolikauri	Homogeneous partially organic vineyard, scarce wild vegetation in surroundings.
	Rokhi	5	N42.116746° E42.720127° 112m a.s.l	Grapevine: Tsolikauri	Homogeneous vineyard, scarce wild vegetation in surroundings. Pesticide application scheme proposed by nfa.gov.ge.
	Sazano	5	N42.190810° E43.05072° 196m a.s.l.	Grapevine: Tsolikauri	Small scale vineyard surrounded by some fruit trees and wild vegetation including elderberry and blackberry. Pesticide application scheme proposed by nfa.gov.ge.
Kakheti	Kondoli	5	N41.960772° E45.596703° 370m a.s.l	Grapevine: Saperavi and Cabernet Sauvignon	Homogeneous vineyard with fruit trees (plum, peach) around. Pesticide application scheme proposed by nfa.gov.ge.
	Kurdgelauri	5	N41.95217° E45.529221° 427m a.s.l	Grapevinee: Rkatsiteli	Vineyard surrounded by wild vegetation mostly represented by blackberry. Pesticide application scheme proposed by nfa.gov.ge.

Region	Monitoring Location	Number of traps	GPS coordinates and altitude	Key crop and dominant cultivar	Site description
Kakheti	Mukuzani	5	N41.810777° E45.711029° 512m a.s.l.	Grapevine Saperavi	Vineyard surrounded by wild vegetation mostly represented by blackberry and other perennial herbs. Pesticide application scheme proposed by nfa.gov.ge.
	Shashiani	5	N41.822433° E45.6679821° 669m a.s.l	Strawberry: Kakheti 1	Strawberry field surrounded by wild vegetation mostly represented by blackberry, other perennial herbs and mulberry. Pesticide application scheme proposed by nfa.gov.ge.
Kartli	Agara	5	N42.024877° E43.796545° 646m a.s.l.	Strawberry: Fortuna	Homogeneous strawberry field with almost no wild vegetation in surroundings. Drip irrigation. Pesticide application scheme proposed by nfa.gov.ge.
	Apnisi	5	N41.995626° E43.900884° 662 m a.s.l.	Cherry: Lapins	Cherry orchard with adjacent apples. Drip irrigation. Pesticide application scheme proposed by nfa.gov.ge.
	Kvenatkotsa	5	N42.045033° E 43.831335° 639 m a.s.l	Cherry: Lapins	Homogeneous cherry orchard, almost no any wild vegetation in surroundings. Drip irrigation. Pesticide application scheme proposed by nfa.gov.ge.
	Skra	5	N41.988408° E43.995987° 618m a.s.l	Cherry: Regina	Cherry orchard with some wild vegetation around
Samegrelo	Ingiri	5	N42.471770° E41.797044° 50m a.s.l	Blueberry: Legacy	Homogeneous blueberry plantation, wild vegetation in surroundings including elderberry. Drip irrigation. Pesticide application scheme proposed by nfa.gov.ge.
	Narazeni	5	N42.422460° E41.923300° 131m a.s.l.	Blueberry: Legacy	Homogeneous blueberry plantation, wild vegetation in surroundings including elderberry. Drip irrigation. Pesticide application scheme proposed by nfa.gov.ge.
	Rukhi	5	N42.534009° E41.879254° 133m a.s.l	Blueberry: Legacy	Homogeneous blueberry plantation, wild vegetation in surroundings including elderberry. Drip irrigation. Pesticide application scheme proposed by nfa.gov.ge.

at each site with 4 traps placed at the edges and one in the middle of the field as recommended by the producer. They were installed at the height of 1-1.5 m from the ground. The traps were placed at the shaded areas of the canopy and were checked for the SWD presence once a week. Lures in each trap were changed once per month as recommended by the producer company. In total, for twenty-three study sites, 95 SWD

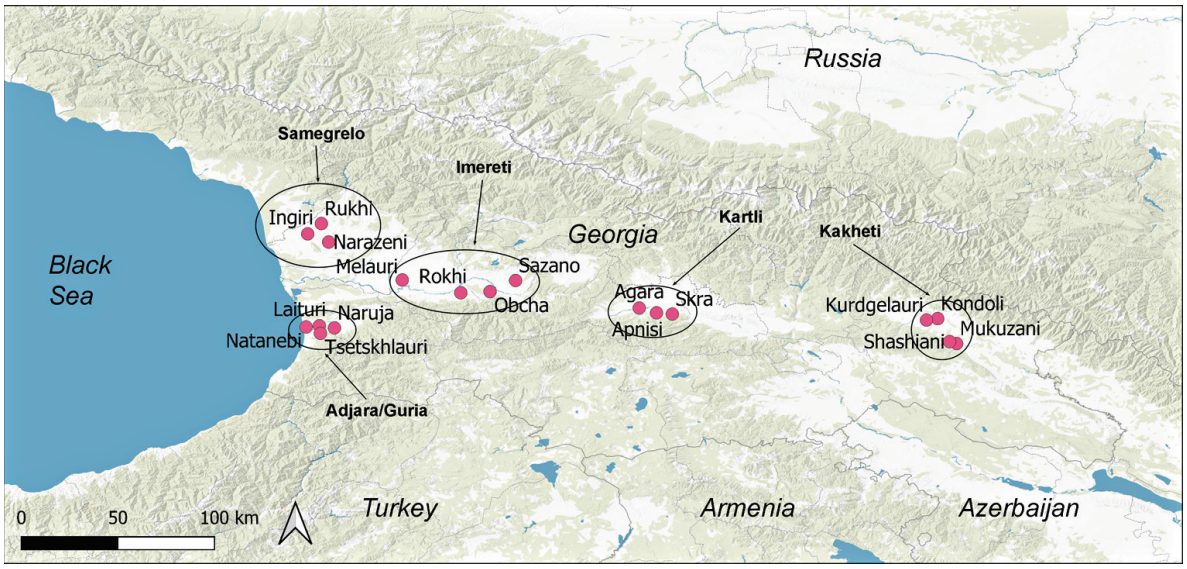

Figure 1. Map showing the distribution of study locations of *Drosophila suzukii* in five major agricultural regions of Georgia (extracted from Murvanidze *et al.*, 2022).

traps were used.

Temperature data for each region were retrieved from local weather stations. Daily minimum, mean and maximum temperature data were retrieved from 1st January 2021 to October 30st 2022. Degree-days (DD) were calculated using 7.2°C as a lower development threshold (Tochen *et al.*, 2014). Cumulative daily DD totals were calculated for each site until the end of monitoring in the end of October.

The captured flies were extracted from traps using a fine painting brush and stored in 95% alcohol for further sorting, identification and morphological analyses. The flies were identified and sex-separated using stereoscope UNITRON Z850 to confirm presence of *D. suzukii*. Identified flies were counted and separated into the male and female adult individuals. After identification, the sampled material was stored in 70% alcohol and voucher specimens are stored to the insect collection of the Agricultural University of Georgia.

Data analysis

For the population dynamics, the average SWD captures was calculated per week, per region for major crops, namely blueberry for Guria and Samegrelo, grapevine for Imereti and Kakheti, and cherry for Kartli region. To estimate the sex-ratio of SWD, the weekly average numbers of male and female individuals were used.

The effect of the year, season, region and crop species on the SWD captures was analyzed independently using a "linear mixed effects model" with random factors of trap and date (of weekly monitoring); the function "lmer" of the lme4 package (Bates *et al.*, 2014) and ANOVA of the "car" package (Fox and Weisberg, 2018) were used. Data on *D. suzukii* captures were log transformed before the analysis to improve homogeneity of variances. All analyses were carried out using R version 4.3.2 (R Core Team 2022).

Results

The first SWD adults in all study regions were observed in the beginning of May. The first SWD flies were captured in Guria and Samegrelo regions when average daily high temperatures exceeded 13°C and 10°C (172 and 246 DD7.2 respectively). In Imereti region first flies showed up on 21 June (745 DD7.2) in 2021 season, while in 2022, they were already present in the field on 15 May (254 DD7.2). In Kakheti region they first appeared

on 28 June in 2021 (954.5 DD7.2) and on 14 June in 2022 (672 DD7.2) and in Kartli they were trapped on 17 July in 2021 (957.3 DD7.2) and on 22 June (563.4 DD7.2) in 2022.

In blueberry fields in Guria and Samegrelo regions, the number of SWD captures increased in the end of July and the beginning of September (Fig. 2). In vineyards, the increase of pest abundance started with the beginning of ripening of grapes from August. However, in the Imereti region it dropped in September, while in Kakheti the numbers continued to increase until October (Fig. 2).

The year was a significant factor for overall population of SWD captures (DF = 1, p<0.001, Table 2) which were lower in 2021 compared to 2022 (Fig. 3, Table 2). The region significantly affected the captures of SWD (DF = 4, p < 0.001, Table 2). The effect of time was different in each region: from 2021 to 2022, the number of individuals increased by 365% in Imereti, by 200% in Guria, by 150% in Kartli, by 1600% in Kakheti and by 4380% in Samegrelo (Table 3, Fig. 4).

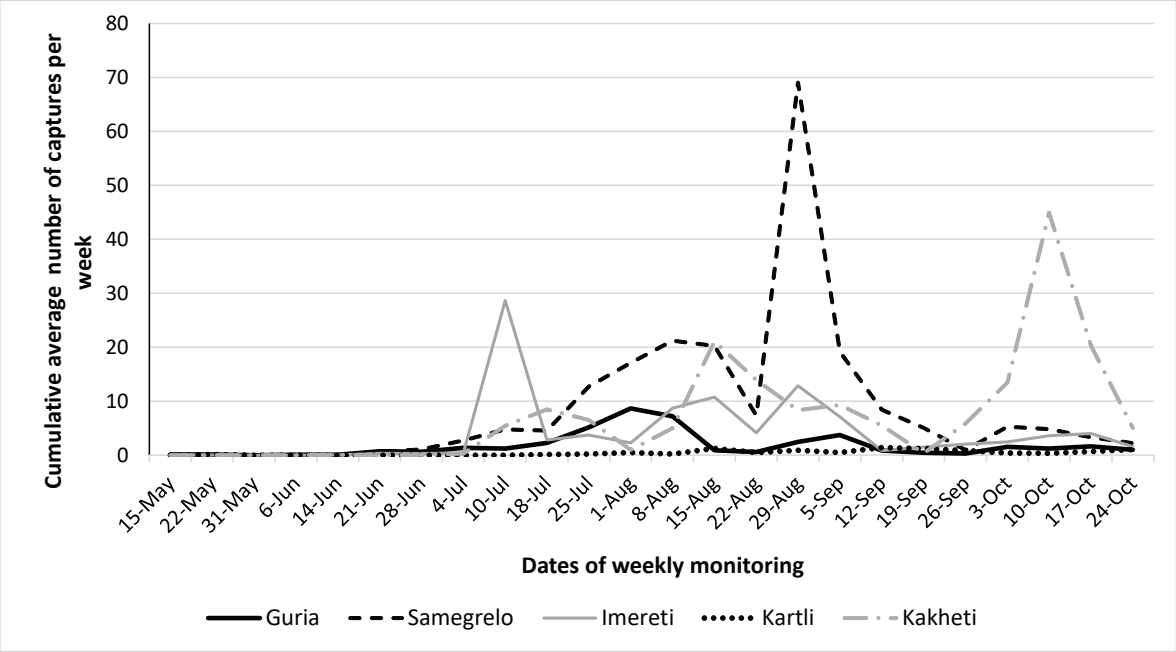

Figure 2. Fluctuation of *Drosophila suzukii* population in Guria, Samegrelo, Imereti, Kartli and Kakheti agricultural regions of Georgia. Abundance represents average of weekly trap captures of adult *D. suzukii* in the years 2021 and 2022.

Table 2. Effect of year, season, region and crop on the abundance of *Drosophila suzukii* three crops (blueberry, strawberry, cherry, wine grapes) in five major agricultural regions of Georgia (Guria, Samegrelo, Imereti, Kartli and Kakheti). Parameter estimates of linear mixed effects model with the random factor trap and date of observation with fixed factors year, season, region and crop. Data of *D. suzukii* abundance were log transformed before the analysis (Significance codes: 0 '***'0.001 '**' 0.01'*' 0.05'.' 0.1''1).

	Chisq	DF	Pr (> Chisq)
Year	141.5	1	<0.001***
Season	61.7	5	<0.001***
Crop	8	3	<0.05 *
Region	27.8	4	<0.001***

The crop (blueberry, strawberry, vine grapes, cherry) was a less significant driver of pest abundance (DF = 4, p<0.05, Table 2), with lower captures in cherry orchards compared to blueberry, strawberry and grapes (Fig. 5).

The season was another significant contributor (DF = 5, p < 0.001, Table 2) to the pest density. While the pest population remained low during the 2021 monitoring period, in 2022, the number of captures were

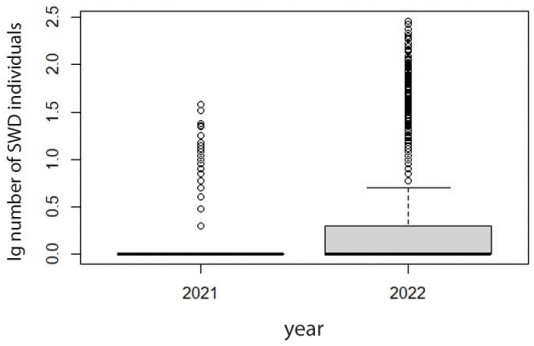

Figure 3. Population of *Drosophila suzukii* (average number of individuals after log transformation of data) across five major agricultural regions of Georgia during the years 2021, 2022.

higher in autumn and summer compared to spring (Fig. 6).

Sex ratio between the average numbers of male and female individuals of *D. suzukii* was almost equal both, in 2021 and 2022 years (Fig. 7A). The same pattern was observed on a regional basis with the exception of Samegrelo and Kakheti regions where number of females exceeded the number of males in 2022 (Fig. 7B).

Discussion

As mentioned above, the presence of *D. suzukii* in Georgia was first detected in 2017 (Japoshvili *et al*., 2018) and by 2021 it was already distributed countrywide (Murvanidze *et al*., 2022). However, the initial date and the route of the pest invasion remain unknown. The preliminary, small-scale monitoring in blueberry and strawberry fields (Guria region) conducted in 2020 (in the same locations as in the current study) from early July to the end of October, showed presence of SWD individuals started in early July (own unpublished data). These and continued observations confirm the existence of well-established population of SWD in this region and lead us to assume that the pest invaded Georgia much earlier prior to the first detection in 2017. According to early simulation models (Cini *et al*., 2012) spread of the pest was predicted all over Europe, towards more humid areas compared to the dry Mediterranean environments. Indeed,

Table 3. Mean population of *Drosophila suzuki* in five major agricultural regions of Georgia (Guria, Samegrelo, Imereti, Kartli and Kakheti) during 2021 and 2022.

Sites		Mean SWD ± Stdev	
		2021	2022
Kakheti	Kondoli	0.64 ± 0.29	9.87 ± 4.71
	Kurdgelauri	1.38 ± 0.32	16.07 ± 4.94
	Shashiani	2.25 ± 0.59	28.20 ± 5.57
	Mukuzani	0.71 ± 0.14	30.96 ± 11.59
Kartli	Skra	0.27 ± 0.08	0.81 ± 0.22
	Apnisi	1.13 ± 0.23	1.99 ± 0.70
	Agara	0.16 ± 0.08	0.61 ± 0.18
	Kvenatkoca	0.01 ± 0.01	0.59 ± 0.29
Imereti	Melauri	1.45 ± 0.38	13.78 ± 5.47
	Obcha	1.46 ± 0.42	5.24 ± 1.72
	Rokhi	0.74 ± 0.19	2.64 ± 1.13
	Sazano	2.43 ± 0.81	6.63 ± 1.33
Samegrelo	Rukhi	0.28 ± 0.11	6.92 ± 2.20
	Ingiri	0.46 ± 0.22	30.75 ± 10.89
	Narazeni	1.43 ± 0.48	23.35 ± 10.63
Guria	Tsetskhlauri	0.37 ± 0.08	1.81 ± 045
	Natanebi	0.54 ± 0.14	0.83 ± 0.27
	Naruja	2.28 ± 0.65	7.17 ± 2.81
	Laituri	0.33 ± 0.09	0.67 ± 0.20

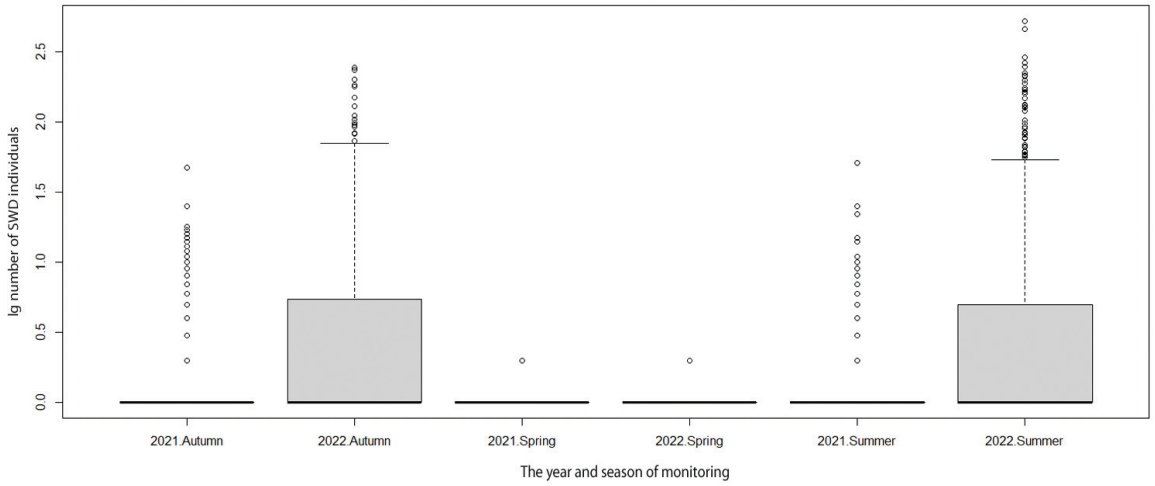

Figure 4. Population of *Drosophila suzukii* (average number of individuals after log transformation of data) in different seasons (spring, summer, autumn) across five major agricultural regions of Georgia during the years 2021 and 2022.

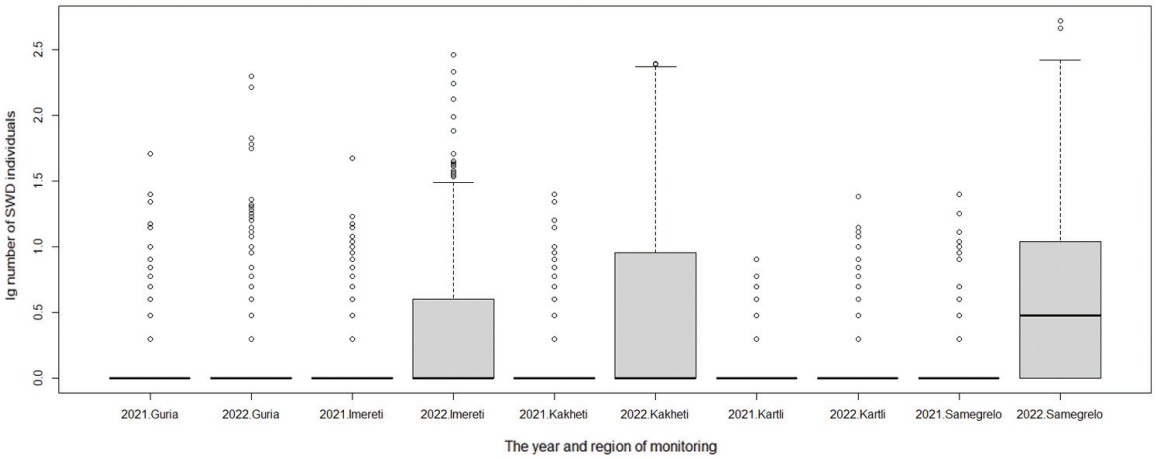

Figure 5. Population of *Drosophila suzukii* (average number of individuals after log transformation of data) in the agricultural regions Guria, Samegrelo, Imereti, Kartli and Kakheti, in Georgia, during the years 2021 and 2022.

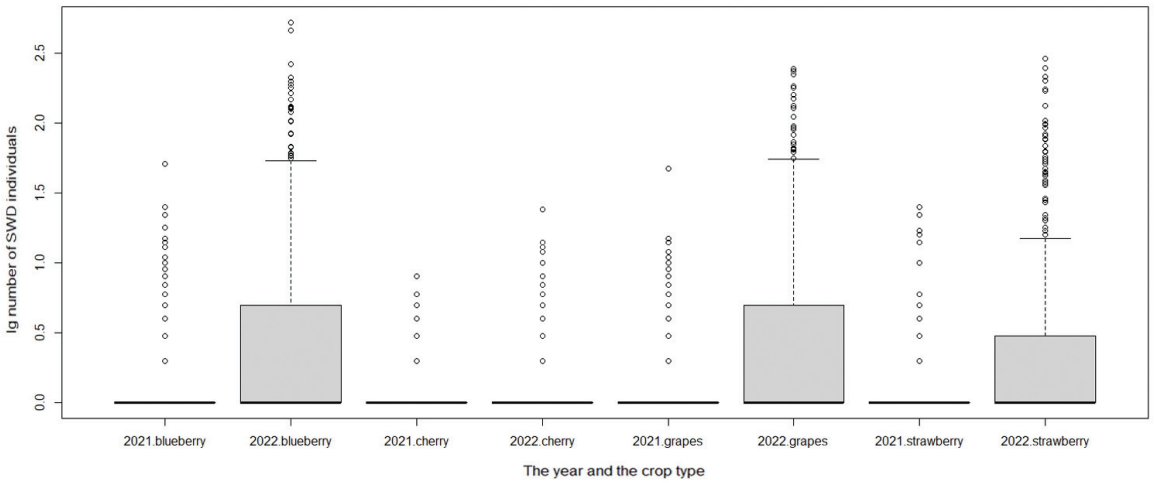

Figure 6. Population of *Drosophila suzukii* (average number of individuals after log transformation of data) in different crops (blueberry, strawberry, cherry, grapevine) in Georgia during the years 2021 and 2022.

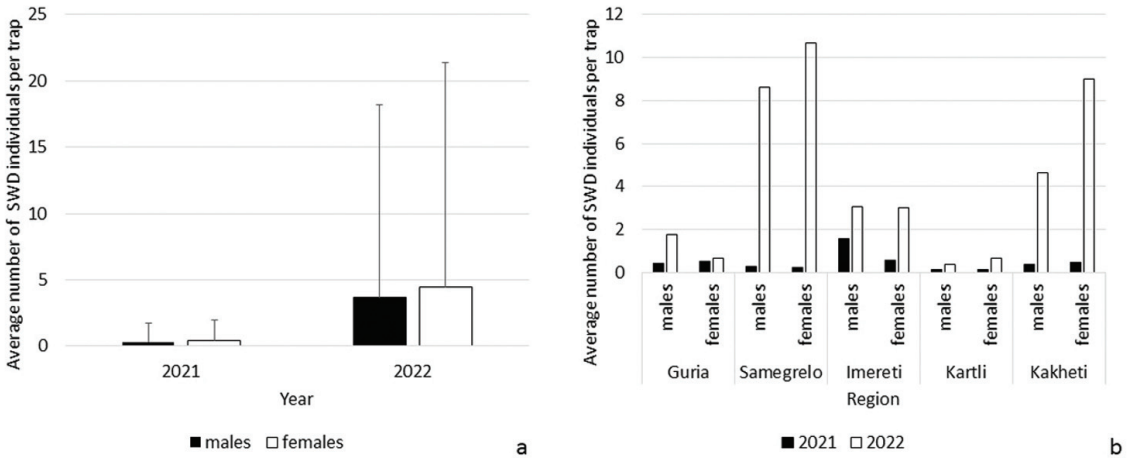

Figure 7. Average number of male and female individuals of *Drosophila suzukii* captured per trap a) in years 2021 and 2022; b) in the regions Guria, Samegrelo, Imereti, Kartli and Kakheti, Georgia during the years 2021 and 2022.

our studies confirm spread of SWD from the Western part of the country, areas with humid subtropical climate towards the East, regions with dry continental climate.

The pest population started to build up from July and continued to increase through August and September. In wine producing regions, SWD population increased in autumn, close to the vine grapes harvesting season. These observations are in accordance with the phenological records provided in regions of a similar latitudinal range (Joshi *et al*., 2016; Walton *et al*., 2019; Swoboda-Bhattarai and Burrack, 2020). This allows to state that the early season crops are out of the risk of infestation (Joshi *et al*., 2016).

The year was a significant contributor showing a population increasing pattern from 2021 to 2022. However, the difference was less significant for Guria region, where pest was first detected. This finding supports our previous assumption about the initial invasion of SWD from Southwestern part of the country. We speculate that the pest entered Georgia from neighboring country - Turkey, where its presence was first recorded in 2014 in Erzerum, close to Guria-Achara part of Georgian border (Orhan *et al*., 2016). On the other hand, *D. suzukii* was first recorded in Russia in 2017 in Sochi (Bienkowsky and Orlova - Bienkowskaya 2020), which is close to Abkhazeti and Samegrelo regions of Northwestern border of Georgia. As the abundance of *D. suzukii* in Samegrelo orchards was significantly lower in 2021 compared to 2022, we assume that 2021 was the establishment year for the pest followed by the dramatic increase of population in 2022. In cherry orchards and the strawberry field located in Kartli region, the pest population remained low. These locations represent homogeneous cherry plantations without wild vegetation that could serve as alternative host for the pest.

The suggested pathway of pest distribution from west to east Georgia is further supported by the dates of first observation events: in Imereti region during the 2021 season, the presence of SWD individuals was first detected on 21 June, while in 2022, the pest was already present on 15 May. The same applies for Eastern Georgia - Kartli (first observation in 2021 – 10 July, in 2022 – 15 May) and Kakheti (first observation in 2021 – 28 June, in 2022 – 14 June). This pattern is an indicator of unstable, still establishing population, while close dates of first observations confirm adaptations of the pest to specific climate conditions (Drummon *et al*., 2019).

Crop type had a less significant effect on the pest distribution and abundance. Indeed, we could observe an increase of the pest population in blueberry plantations in the end of the summer when the main crop was long harvested. These plantations were

surrounded with wild alternative susceptible host plants for SWD such as blackberry (*Rubus* spp.) and elderberry (*Sambucus nigra* L.). In vineyards, the increase of pest population coincided with ripening of grapes, however, the highest increase in abundance in vineyards was observed in early October, when harvest was already finished. Remaining over ripe grapes together with surrounding blackberries may have supported the presence of the pest. The importance of alternative hosts to maintain the population of *D. suzukii* has been reported by a number of other studies (Asplen *et al*., 2015; Arnó *et al*., 2016; Drummond *et a*l., 2019).

The proportion of male : female individuals of *D. suzukii* individuals captured in the traps supported 1:1 sex ratio for Guria, Imereti and Katli region in both years, while in Samegrelo and Kakheti proportion of females was higher in 2022. Drummond *et al.* (2019) reported that drop of proportion of male individuals over seven years of observations, could affect overall population size in a long-term period, although, he indicated that this pattern was probably due to bacterial (*Wolbachia*) infestation. Similarly, different factors, including crop phenology or diseases could affect patterns of sex ratio which therefore might not serve as a reliable indicator of future population growth.

Provided investigation in current work confirms well established population of *D. suzukii* all over the country and in all susceptible crops. There is a rapid increase of the pest population and widening dispersal within two observation years, indicating the urgent need of evaluation of the applied management strategies to prevent economic losses. Studies in other berry production countries report 20% or higher yield losses if SWD is left uncontrolled (Bolda *et al*., 2010, Goodhue *et al*., 2011; Walsh *et al*., 2011).

At the same time the importance of preventative control tactics is widely acknowledged (Lee *et al*., 2011; Leach *et al*., 2017; Knapp *et al*., 2020). None of such strategies for *D. suzukii* control are elaborated in Georgia so far. Pesticide applications and sanitary procedures are known as immediate solutions, but there is low likelihood of gaining long-term effect. Insecticides used for control of SWD population are broad-spectrum, killing also beneficial insects (Walsh *et al*., 2011; Cini *et al*., 2012; Hoffmann Schlesener *et al*., 2017). The problem is even worse for organic crop producers as there are no species-specific insecticides for *D. suzukii* (Tait *et al*., 2021).

The most important objective in integrated pest management of *D. suzukii* is to monitor the population dynamics of the pest, showing seasonal fluctuation and peek activity (Drummond *et al*., 2019). Various baits for monitoring are available in the market or self-made (Walsh *et al*., 2011). Also, our study showed a clear increase of SWD correlating with the ripening of alternative crops such as blackberry or elderberry in the surroundings of the crop field. Rapid harvest of crops and removal of surrounding alternative host plants that maintain pest population when the crop plant is not available, is crucial to maintain population below the threshold level as reported by literature (Cini *et al*., 2012; Leach *et al*., 2017). Maintenance of spoiled fruit or other plant residues, as well as storage containers in the field is another possible shelter for the pest as detected in strawberry field in Guria region (trap close to such area showed higher captures of flies compared to other traps in Natanebi) and confirmed by other studies (Cini *et al*., 2012; Leach *et al*., 2017).

Based on our findings, integrated pest management of *D. suzukii,* in our perception, should include the following major components: continuous monitoring of pest population using commercially available or hand-made traps; minimize the use of broad-spectrum insecticides in cases of increased captures in traps; sanitation procedures including removal of and of any plant residues, compost, containers in field and of alternative hosts surrounding crop fields/orchards.

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Παρακολούθηση πληθυσμού και αξιολόγηση της κατάστασης του νέου χωροκατακτητικού εντόμου, *Drosophila suzukii* (Matsumura) (Δίπτερα: Drosophilidae), σε διάφορα συστήματα καλλιέργειας της Γεωργίας (Sakartvelo)

G. Japoshvili, M. Murvanidze, N. Inasaridze, N. Meskhi, Z. Lipartia και L. Namicheishvili

Περίληψη Πραγματοποιήθηκε παρακολούθηση της διακύμανσης του πληθυσμού και της κατανομής της κηλιδόπτερης δροσόφιλας *Drosophila suzukii* (Matsumura, 1931) σε τέσσερις ευπαθείς καλλιέργειες (μύρτιλο, φράουλα, κεράσι, οινοποιήσιμα σταφύλια) σε πέντε κύριες γεωργικές περιοχές της Γεωργίας (Guria, Samegrelo, Imereti, Kartli and Kakheti) κατά τα έτη 2021 και 2022 με στόχο τη μελέτη του μεγέθους του πληθυσμού και την εκτίμηση της κατάστασης του εντόμου. Η παρακολούθηση του πληθυσμού διεξήχθη σε τέσσερις τοποθεσίες κάθε περιοχής μελέτης σε εβδομαδιαία βάση από τον Μάιο έως τον Νοέμβριο, με τη χρήση παγίδων PHEROCON® SWD με δόλωμα ευρέως φάσματος PHEROCON® SWD

PEEL-PAK™. Η έρευνα έδειξε σημαντική αύξηση του πληθυσμού από το 2021 έως το 2022 στις περισσότερες από τις τοποθεσίες που μελετήθηκαν. Αύξηση πληθυσμού διαπιστώθηκε σε όλους τους οπωρώνες εκτός από τις κερασιές. Η απουσία εναλλακτικών φυτών ξενιστών σε γειτνίαση με την καλλιέργεια έχει κρίσιμη σημασία για την προστασία της καλλιέργειας από την επέκταση του εντόμου. Το καλλιεργούμενο είδος δεν είχε σημαντική επίδραση στο *D. suzukii*. Ο πληθυσμός του εντόμου ήταν σημαντικά μεγαλύτερος το καλοκαίρι και το φθινόπωρο σε σύγκριση με την άνοιξη, πιθανώς λόγω της ωρίμανσης των καρπών εναλλακτικών καλλιεργειών όπως το βατόμουρο και ο σαμπούκος. Η αναλογία φύλου μεταξύ αρσενικών και θηλυκών ατόμων ήταν σχεδόν 1:1 και παρέμεινε σταθερή κατά τη διάρκεια της διετούς περιόδου μελέτης σε όλες τις περιοχές. Εκτιμούμε ότι το *D. suzukii* εισήλθε στη χώρα από το νοτιοδυτικό τμήμα της και επεκτάθηκε προς τα ανατολικά. Μέχρι στιγμής στη Γεωργία δεν έχουν αναπτυχθεί στρατηγικές για τον έλεγχο του *D. suzukii*. Επεμβάσεις με αποτελεσματικά φυτοπροστατευτικά σκευάσματα με βάση την παρακολούθηση το εντόμου καθώς και μέτρα υγιεινής που περιλαμβάνουν απομάκρυνση των εναλλακτικών φυτών ξενιστών και τυχόν υπολειμμάτων της καλλιέργειας από το χωράφι είναι αναγκαία για να αποφευχθεί πιθανή πληθυσμιακή έξαρση του εντόμου.

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