

Entomotoxic Activity of the Extracts from the Fungus, *Alternaria tenuissima* and Its Major Metabolite, Tenuazonic Acid

Dilara Salimova¹, Anna Dalinova¹, Vsevolod Dubovik¹, Igor Senderskiy¹,
Elena Stepanycheva¹, Oksana Tomilova², Qiongbo Hu³, Alexander Berestetskiy^{1,*}

¹ Department of Phytotoxicology and Biotechnology, All-Russian Institute of Plant Protection, Podbelskogo shosse, 3, Pushkin, Saint-Petersburg 196608, Russian Federation;

d.salimova@vizr.spb.ru (D.S.); adalinova@vizr.spb.ru (A.D.); vdubovik@vizr.spb.ru (V.D.); senderskiy@mail.ru (I.S.); estepanycheva@yandex.ru (E.S.); aberestetskiy@vizr.spb.ru (A.B.)

² Institute of Systematics and Ecology of Animals SB RAS, Frunze Str. 11, 630091 Novosibirsk, Russia; toksina@mail.ru

³ Key Laboratory of Bio-Pesticide Innovation and Application of Guangdong Province, College of Plant Protection, South China Agricultural University, Guangzhou 510642, China; hqbscau@scau.edu.cn

* Correspondence: aberestetskiy@vizr.spb.ru; Tel.: +7 (812) 476-6838

Abstract

Study of fungal antibiotics in their competitive interactions with arthropods may lead to development novel biorational insecticides. Extracts of *Alternaria tenuissima* MFP253011 obtained by various methods showed a wide range of biological activity, including entomotoxic properties. Analysis of their composition and bioactivity allowed to reveal several known mycotoxins and unidentified compounds that may be involved in entomotoxic activity of the extracts. Among them, tenuazonic acid (TeA), which was the major component of the *A. tenuissima* extracts, was found the most likely to have larvicidal activity against *Galleria mellonella*. In the intrahaemocoel injection bioassay, TeA was toxic to *G. mellonella* and of *Zophobas morio* with LT₅₀ 6 and 2 days, respectively, at the level of 50 µg/larva. Administered orally, TeA inhibited growth of *G. mellonella* larvae and caused mortality of *Acheta domesticus* imagines (LT₅₀ 7 days) at a concentration of 250 µg/g of feed. TeA showed weak contact-intestinal activity against the two phytophages, *Tetranychus urticae* and *Schizaphis graminum*, causing the 12 and 40% of mortality at a concentration of 1 mg/mL. TeA was cytotoxic to Sf9 cell line (IC₅₀ 25 µg/mL). Thus, model insect *G. mellonella* and cell line Sf9 could be used for a further toxicological characterization of TeA.

Keywords: *Alternaria tenuissima*, extract, bioassays, PCA, tenuazonic acid, *Galleria mellonella*, *Zophobas morio*, *Acheta domesticus*, *Tetranychus urticae*, *Schizaphis graminum*, Sf9

1. Introduction

Herbivores and phytopathogens inhabit the same ecological niches using plants as a food source and, therefore, can compete for the substrate. In this regard, some phytopathogenic microorganisms are assumed to produce metabolites that directly (due to entomotoxic or repellent action) or indirectly (through induced plant responses, suppressed immunity and the symbiotic

microflora of insects) affect the fitness of arthropods. The study of the antagonistic effect of phytopathogenic microorganisms on viability, development and fertility of arthropods is important for the development of methods to control phytophagous insects and vectors of human and animal diseases [1].

Among phytopathogenic fungi, *Alternaria* genus species have a potential for the production of entomotoxic secondary metabolites. For instance, extracts of about 20% of tested isolates of nine *Alternaria* species showed aphicidal activity against the vetch aphid (*Meurgura viciae*). The aphid was the most sensitive to organic extracts from cultures of *A. saponariae*, *A. japonica*, *A. penicillata*, *A. papavericola*, and *A. tenuissima* [2]. An ethanolic extract from the mycelium of *A. papavericola* (= *Brachycladium papaveris*) caused mortality of *M. viciae* comparable to the botanical insecticide NeemAzal [3]. Extracts from *A. alternata* cultures inhibited acetylcholinesterase and also exhibited insecticidal and immunosuppressive activity against the cotton leafworm (*Spodoptera litura*) [4,5]. Extracts from *A. destruens* cultures that inhibited the activity of α -glucosidase were toxic to *S. litura* with LD₅₀ of about 2 mg/g feed [6].

A number of entomotoxic compounds had been purified from cultures of several *Alternaria* species. *A. brassicae* was found to produce depsipeptide phytotoxins, some of which (for example, destruxin B) demonstrates insecticidal properties [7,8]. Methyl-3,8-dihydroxy-6-methyl-4-chloro-9-oxo-9H-xanthene-1-carboxylate and chloromonilinic acid B isolated from *A. sonchi* strongly affected (75% mortality) the common wheat aphid (*Schizaphis graminum*) at a concentration of 1 mg/mL [9]. Altenuene produced by *A. alternata* led to 70% mortality of *S. litura* larvae at 5 mg/g feed [10]. Tenuazonic acid produced by some *Alternaria* species was toxic for the first instar larvae of the common green bottle fly (*Lucilia sericata*) (LD₅₀ 120 μ g/mL) [11].

Small-spored *Alternaria* spp. may be of special interest as possible producers of entomotoxic metabolites. These species have often been isolated from cadavers or hibernating stages of arthropods. They also demonstrate entomopathogenic properties [12–14]. In particular, the strains of the fungus, preliminary identified as *A. infectoria*, infected eggs, larvae, and imago of the fig wax scale (*Ceroplastes rusci*) [15]. Interestingly, *A. alternata* strains pathogenic for various aphid species did not infect insects from other orders suggested the production of aphid-specific toxins by the fungus [16,17]. At the same time, small-spored *Alternaria* spp. of the section *Alternaria* (*A. alternata*, *A. arborescens*, *A. gaisen*, and *A. tenuissima*) being widespread on various plant substrates as saprotrophs and weak pathogens [18] can indirectly affect various arthropods by production of phytotoxins or/and plant immunomodulators [19,20]. Therefore, despite the small-spored *Alternaria* spp. are able of infecting arthropods, the entomotoxic properties of their secondary metabolites are still poorly understood.

The earlier characterized strain MFP253011 of *A. tenuissima* [21] was chosen as the object of this study. The aim of this work was to reveal and characterize the determinants of *A. tenuissima* MFP253011 entomotoxicity. For this purpose, 1) the fungal cultures were obtained on five liquid and solid substrates to extract the metabolites with two solvents and, then, to evaluate the spectrum of their bioactivity, 2) the composition of the extracts was analyzed in relation with the bioactivity to reveal entomotoxicity determinants and their possible indirect effects; 3) tenuazonic acid (TeA), which was found to be a main component of extracts showing anti-insectan properties, was assayed against various species of model (*Galleria mellonella*) and feed (*Zophobas morio* and *Acheta domesticus*) insects, as well as phytophages (*Schizaphis graminum* and *Tetranychus urticae*).

2. Materials and Methods

2.1. Fungal strain and fermentation

The strain MFP253011 gifted by Dr. Philipp Gannibal (Laboratory of mycology and phytopathology at All-Russian Institute of Plant Protection, Saint-Petersburg, Russia) and identified as *A. tenuissima* (Kunze) Wiltshire [21] was used in this study. The fungal culture was preserved on standard potato-dextrose agar (PDA) at a temperature of 5°C. To obtain extracts, the fungus was grown in 1-L Erlenmeyer flasks with 300 mL of a liquid medium. Four standard liquid synthetic and semisynthetic nutrient media varied by carbon and nitrogen sources were used: modified Czapek media (MCM), M1D, YMG and Sabouraud media (SAB). Their composition (g per L of deionized water, pH 6) is following: MCM (glucose – 20, NaNO₃ – 2, KH₂PO₄ – 1, MgSO₄ – 0.5, KCl – 0.5, thiamine 0.1×10⁻³, biotin – 0.005×10⁻³), M1D (sucrose – 45, ammonium tartrate – 7.5, MgSO₄ × 7 H₂O – 5.25, Ca(NO₃)₂ – 0.45, KNO₃ – 0.15, KCl – 0.15, NaH₂PO₄ – 0.03, FeCl₃ × 6 H₂O – 0.003, ZnSO₄ × 7 H₂O – 0.0375, H₃BO₃ – 0.003, r KI – 0.015, MnSO₄ – 0.75), YMG (glucose – 20, malt extract – 10, yeast extract – 4), SAB (glucose – 40, peptone – 10).

The fungal culture was also obtained on a solid substrate: 100 g of pearl barley (PB), 60 mL of water in 500-mL Erlenmeyer flasks. All the media used in this study were sterilized by autoclaving at 121°C for 20 minutes. Agar blocks (5 mm in diameter) cut off the edge of one-week *A. tenuissima* colonies obtained on PDA at 24°C were used as seed inoculum (two blocks per flask). Incubation of the fungus was carried out at a constant temperature of 24°C in the dark: without agitation for three weeks for the liquid cultures, while the solid cultures were grown for two weeks and shaken once a day to improve aeration and prevent clumping. Three biological replicates were used for each culture medium.

2.2. Extraction of secondary metabolites

Culture filtrates of *A. tenuissima* (300 mL each) were adjusted to pH 7 with 0.1N NaOH and extracted with dichloromethane (DCM) (2 × 150 mL). Then remaining aqueous phases were adjusted to pH 3 with formic acid and repeatedly extracted with ethyl acetate (EtOAc) (2 × 150 mL). The dry solid culture was blended and extracted repeatedly with 300 mL of a mixture of acetone and water (50:50, v/v). After evaporation of the organic solvent, water phase was successively extracted with hexane and EtOAc, twice for each solvent. The extracts were dried over anhydrous sodium sulfate and evaporated to dryness under vacuum at 40 °C.

2.3. Bioassays

2.3.1. Entomotoxic activity

The toxicity of *A. tenuissima* extracts was evaluated against spring green aphid (*Schizaphis graminum*) and greater wax moth (*Galleria mellonella*) obtained from Laboratory of biological control at All-Russian Institute of Plant Protection. *S. graminum* was reared on wheat seedlings at 25±1°C and 65±5% relative humidity, while *G. mellonella* was kept in the dark at 30°C in plastic boxes with perforated lids filled with artificial feed (90 g corn grits, 40 g wheat flour, 50 g milk powder, 10 g yeast, 50 g glycerol, 50 g beeswax, 50 mL water). Prior bioassays, the extracts samples were dissolved in ethanol, and then the solutions were adjusted with water to extract concentration of 5 mg/mL while the final concentration of ethanol was 5% (v/v).

The contact-intestinal aphicidal bioassay was described in detail earlier [22]. Briefly, filter paper disks (4 cm in diameter) placed on the bottom of a Petri dish were moistened with 0.5%

extracts (250 μL per disk or 1 mg/dm^2). Segments of wheat leaves (2 cm length) were dipped in the same test-solutions and were placed on the filter paper. In the control treatment, 5% aqueous ethanol (v/v) was used. Twenty aphids were transferred to each Petri dish. Mortality percentage of tested insects was recorded 24 h post treatment at above mentioned conditions. Four replicates were made for each extract.

For evaluation of the acute-contact larvicidal activity of the extracts, the injection method was used as described previously [23] with slight modifications. Briefly, 10 μL of 0.5% extracts were injected into the hemocoel via third segment of IV–V instar *G. mellonella* larvae (190–230 mg each) with Hamilton syringe. As a control treatment, 5% aqueous ethanol (v/v) was used. After the extract injection of the larvae were placed into a 90-mm sterile Petri dish supplemented with 2 g of the artificial feed and were incubated in the dark for 10 days to record mortality daily. Twenty *G. mellonella* larvae were tested per treatment, and the experiment was repeated twice.

2.3.2. Phytotoxic activity

The phytotoxic activity of the extracts was assayed using the previously described method [24]. Leaf discs (1 cm in diameter) of perennial sowthistle (*Sonchus arvensis*) and leaf segments (2 cm length) of wheat (*Triticum aestivum*) were placed in a wet chamber and accurately punctured by a sharp needle. A 10 μL droplet of 0.5% extract prepared in 5% ethanol was placed above the puncture. In the control treatment, 5% aqueous ethanol was used. The phytotoxic activity was determined as the diameter or length of necrotic lesions (for sowthistle and wheat, respectively) 48 h of exposure at 24°C and 12-h photoperiod. Six leaf disks/segments were used for each treatment.

2.3.3. Cytotoxic activity

The cytotoxicity activity of *A. tenuissima* extracts was assayed on the Sf9 cell line (ECACC 89070101) originated from the ovarian tissue of the fall armyworm (*Spodoptera frugiperda*) that has been maintained in the Laboratory of molecular plant protection at All-Russian Institute of Plant Protection. Samples (10 μL) of assayed extracts dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/mL were added to 890 μL of fresh SF900II culture medium (Thermo Fisher Scientific, Waltham, MA, USA) and 100 μL of a suspension of actively growing cells (1×10^5 cells/ mL , viability $\geq 90\%$) in wells of a 48-well plate. The final concentration of the solvent was 1%. 1% DMSO solution was used as a control treatment. The cells were incubated for 24 h at 27°C, then stained with 0.4% aqueous solution of trypan blue to count the percentage of dead (stained) cells in relation to the total number (at least 50) in several fields of view. Three replicates were used for each extract.

2.3.4. Antimicrobial activity

The antimicrobial activity of *A. tenuissima* extracts was tested against *Bacillus subtilis* NCTC 104000 and *Candida albicans* NCPF 3179 by using the paper-disk agar diffusion assay [25]. The microorganisms were grown on PDA. The samples of assayed extracts were dissolved in acetone and applied to the 6 mm paper discs (Macherey-Nagel, Düren, Germany) at a concentration 500 $\mu\text{g}/\text{disk}$. The treated microbial cultures were incubated at 30°C for 24 h before activity was determined as the radius of the growth inhibition zone in mm. Three replicates were used for each extract.

2.4. Analysis of extracts

Acetonitrile was used to dissolve the extracts samples to a concentration of 5 mg/mL. They were analyzed with Acella high-performance liquid chromatography (HPLC) system with a diode-array detector and TSQ Quantum Access™ triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, USA). The analysis was performed on column Zorbax SB-C18 (Agilent Tech., Santa Clara, CA, USA, pore size 1.8 μm , 4.6 \times 150 mm) with an eluent flow of 250 $\mu\text{L}/\text{min}$, a column temperature of 40°C in a solvent system of acetonitrile–0.1%-formic acid in the gradient mode: acetonitrile concentration increased from 1 to 95% during the first 17 min, remained at 95% of acetonitrile for 3 min; the volume of the injected samples was 2 μL . The samples were analyzed in the positive mode in the scanning range of 100–1000 m/z using heated electrospray ionization. The retention time (t_R), m/z values, UV-spectra of the metabolites were analyzed to compare the chromatographic profiles of the extracts.

The relationship between the composition of extracts (9 major compounds) obtained by different methods (5 media \times 2 extraction solvents) and their biological activity (9 organisms) was assessed by principal component analysis (PCA) with data standardization prior the analysis. Additional data on toxicity extracts to radish and *Paramecium caudatum* were taken from a published research [21].

2.5. Isolation of tenuazonic acid

The submerged fermentation of *A. tenuissima* MFP253011 was conducted in a 7-L bioreactor (Applikon Biotechnology, Delft, Netherlands) containing 4.75 L of YMG medium for 7 days. The 250 mL of starter culture was obtained in a 750 mL-Erlenmeyer flask with the same medium that was inoculated with two agar blocks of the fungus colony on PDA (5 mm in diam) and incubated on a rotary shaker (at 180 rpm) at temperature 24 °C for 7 days. The conditions of submerged fermentation were as follows: incubation temperature 24°C, aeration level 1 vvm, rotation speed 400 rpm, initial pH 5.9 was not maintained at a constant level. Sunflower oil (1%, v/v) was added to the medium as an antifoam before the inoculation.

Extraction of metabolites from culture filtrate was performed as described in the section 2.2. The crude EtOAc extract (1272 mg) was separated on glass column packed with Bio-Beads SX-8 resin (Bio-Rad, Hercules, CA, USA) using a mixture of DCM–EtOAc–formic acid (49.9 : 49.9 : 0.2, v/v) as the eluent, flow rate was 5 mL/min. Resulting TeA-containing fraction II (890 mg) was applied to a cartridge Chromabond C18ec (10 g, Macherey-Nagel, Düren, Germany), the column was eluted sequentially with 40 mL of MeOH–0.1% formic acid mixture (25:75, 50:50, 75:25, 100:0, v/v), yielding five subfractions A–E. The subfraction B (775 mg) was further purified by preparative HPLC to afford TeA (475 mg) as yellow oil (XBridge Prep C18 5 μm , column size 19 \times 250 mm, elution with 30% acetonitrile in 0.1% formic acid, flow rate 24 mL/min, detection 272 nm, t_R 11.2 min). The following chromatographic system was used for preparative HPLC: Quaternary Gradient Module 2545, UV/Visible Detector 2489 and Fraction Collector III (Waters, Milford, MA, USA). ^1H and ^{13}C NMR spectra were recorded at 400 and at 100 MHz, respectively, in CDCl_3 as a solvent on a Bruker AVANCE III 400 MHz spectrometer (Bruker, Karlsruhe, Germany). The solvent residual signal (δ 7.26 ppm) for ^1H NMR spectra and the carbon signal of CDCl_3 (δ 77.16 ppm) for ^{13}C NMR spectra were used as references. ESI-MS spectra were recorded with TSQ Quantum Access spectrometer (Thermo Scientific, Waltham, MA, USA) after HPLC as described in the section 2.4. Spectral and spectroscopy data are given in the Supplementary information. TeA was identified by literature data [26].

Tenuazonic acid ESI-MS: m/z 198.02 $[M + H]^+$; 1H NMR: predominant form: 6.77 br.s (NH), 3.8 d (3.5 Hz), 2.47 s, 1.98 m, 1.40 m, 1.25 m, 1.03 d (7.0 Hz), 0.97 t (7.35 Hz); minor form: 6.65 br.s (NH), 3.97 d (3.8 Hz), 2.52 s, 1.98 m, 1.41 m, 1.31 m, 1.03d (7.0 Hz), 0.97 t (7.35 Hz); ^{13}C NMR: predominant form: 195.4, 184.4, 176.3, 102.5, 67.1, 37.3, 23.7, 19.7, 15.8, 11.7; minor form: 201.1, 189.0, 169.8, 106.0, 63.8, 37.1, 24.0, 20.6, 15.4, 11.7.

2.6. TeA entomotoxicity

2.6.1. Insects and toxins

G. mellonella larvae and *S. graminum* females were prepared as described in 2.3.1. section. *Zophobas morio* laboratory population was reared in trays with wheat bran and fruit peelings. The colony of house cricket (*Acheta domesticus*) was reared in plastic boxes filled with carton pieces and fruit peelings. The population of the two-spotted spider mite (*Tetranychus urticae*) was grown on common bean (*Phaseolus vulgaris*) seedlings. All the arthropods were obtained from the mentioned above Laboratory of biological control.

The samples of TeA were dissolved in ethanol for bioassays as described above. The analytical standard (Sigma-Aldrich, St. Louis, MO, USA) of entomotoxic fungal metabolite, beauvericin, was used as positive controls in some bioassays in the same way as TeA.

2.6.2. Acute-contact larvicidal toxicity

Injection test was performed using the IV-V instar larvae of *G. mellonella* (190–230 mg each) and *Z. morio* (135–150 mg each), the procedure was similar as described in 2.3.1. section. The concentrations of TeA solutions for injection were 0.5, 1, 2 and 5 mg/mL. After the injection test-insects were placed in sterile Petri dishes supplemented with 2 g of feed (the artificial feed for *G. mellonella* and a piece of zucchini for *Z. morio*) and incubated in darkness at 30°C for ten days. The mortality and weight of treated larvae were recorded daily. Five larvae were tested as a group within the experiments, and four replicates were performed.

2.6.3. Oral toxicity

G. mellonella larvae and *A. domesticus* imago were subjected to a diet containing the different concentrations of TeA. Bioassay was conducted by dissolving TeA in acetone and adding onto food samples (the artificial feed for *G. mellonella* and a piece zucchini for *A. domesticus*) at concentrations of 0.25, 1.0 and 2.5 mg/g. The control was treated with the same volume of acetone. The solvent was evaporated from treated feed samples at a room temperature for 45 min. Since the second day of the experiment the feed samples were replaced with fresh ones every two days. Ten larvae of *G. mellonella* were tested as a group within the experiments, and two replicates were performed. Ten *A. domesticus* imagines were tested per replicate, and four replicates were performed. The insects were incubated for 10 days at darkness. The mortality and weight of the insects were recorded daily.

2.6.4. Contact-intestinal toxicity

Aphicidal activity of TeA was tested against wheat aphid as described above (2.3.1) at concentrations 0.5, 1.0 and 2.0 mg/mL (100, 200, and 400 $\mu\text{g}/\text{dm}^2$). The acaricidal effects of TeA was tested against the mite *T. urticae* at the same concentrations similar to a published technique [27]. Common bean leaf discs (diameter 4 cm) were dipped for 2 s in the toxin solutions. After

liquid evaporation the leaf discs were placed in a glass Petri dish (diameter 9 cm) on filter paper moistened with water. Leaf discs in the control group were dipped in 5% ethanol as described above. Twenty *T. urticae* females were introduced to each leaf replicate disc, five replicates were performed.

2.6.5. Cytotoxic activity

Sf9 cells were used for TeA cytotoxicity assessment at the concentration range of 1–100 µg/mL as described in the section 2.3.3. как расстворяли?

2.7. Statistical analysis

Statistical analysis was performed using Statistica 10 (StatSoft, Tulsa, OK, USA) and SigmaPlot 14 (Systat Software, San Jose, CA, USA) software. Data normality was determined by the Shapiro-Wilk W test. The values expressed in percents (%) were transformed (lg or square root) prior the tests. Normally distributed data were analyzed using one- or two-way analysis of variance (ANOVA) depending on experimental setup. Pairwise comparison was performed by Tukey's HSD test at the significance level of $P=0.05$. In the absence of a normal distribution, the data were analyzed by methods of nonparametric statistics: the significance of the factor effects was determined using Kruskal-Wallis H-test (one way ANOVA on ranks); the significance of differences between the median values was determined by the Dunn's post hoc test. The survival rate of arthropods under the influence of TeA was determined by log-rank test with Holm-Sidak adjustment. IC_{50} value representing TeA concentration required to cause a 50% reduction in Sf9 cells viability was determined by SigmaPlot curvilinear regression procedure.

3. Results

3.1. The yield of *A. tenuissima* extracts

The results of two-way ANOVA showed that the composition of the nutrient medium ($F_{3,16}=6.1$, $P=0.01$) and the extraction solvent ($F_{1,16}=79.8$, $P=0.001$) significantly affected the yield of extractive matter (YEM) from the culture filtrate of *A. tenuissima* MFP253011. The interaction of both factors was also significant ($F_{3,16}=4.4$, $P=0.02$). The YEM from the fungal culture filtrate obtained on MCM was almost 3 times lower than those obtained on other liquid media. When sequentially extracted with DCM and EtOAc, the YEM was on average 7 times higher in EtOAc extracts than in DCM extracts. The maximum yield of DCM extracts (about 200 mg/L) was obtained by growing the fungus on SAB medium. YEM of EtOAc extracts was maximal when DMG medium (more than 640 mg/L) was used (Figure 1).

When the fungus was cultured on pearl barley, yield of hexane extracts was 408.3 ± 40 mg/kg, while EtOAc extracts gave 1740.3 ± 49.8 mg/kg.

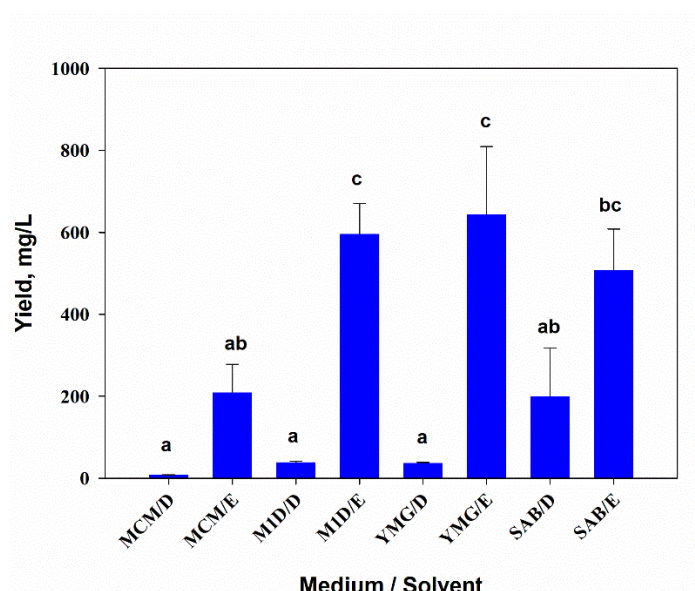


Figure 1. Yield of extractive matter from the filtrate of *Alternaria tenuissima* MFP253011 cultures on various liquid media obtained by successive extractions with dichloromethane (D) and ethyl acetate (E). Means \pm standard deviation marked with the same letter do not differ significantly at the level of $P=0.05$ according to Tukey's HSD test.

3.2. The spectrum of biological activity of *A. tenuissima* extracts

3.2.1. Entomotoxic activity

Results of one-way ANOVA showed that the composition of the substrate and the extraction solvent as a combined factor had a significant ($F_{10,33}=25.37$, $P=0.01$) effect on the aphicidal activity of *A. tenuissima* MFP253011 extracts. The maximal activity against *S. graminum* (>80% mortality 24 h post treatment) was shown by DCM extract from the filtrate of the *A. tenuissima* culture on MID medium. The common wheat aphid was insignificantly (at $P=0.05$) less sensitive to the DCM extract from the filtrate of the *A. tenuissima* culture on SAB (~70% mortality) and EtOAc extract from the solid culture of the fungus (>60% mortality). Hexane extract from the solid culture of *A. tenuissima* also had aphicidal activity (~50% mortality), significantly (at $P=0.05$) different from the control values. The sensitivity of common wheat aphid to other extracts was low ($\leq 25\%$ mortality) and did not differ from the control at $P=0.05$ (Figure 2 A).

The composition of the nutrient medium for growing *A. tenuissima* and the extraction method had a significant effect (one-way ANOVA, $F_{9,10}=7.35$, $P=0.01$) on the toxicity of extracts to *G. mellonella* larvae. The most of the dead larvae were melanized 3–4 days post treatment. No larvae died in untreated control by the 7th day of observations. A high level of larvicidal activity ($\geq 75\%$ mortality 7 days post treatment), which was significantly different from the control at $P=0.05$, was demonstrated by EtOAc extracts from the cultures produced on various liquid (M1D, DMG, Sabouraud's media) and solid substrates. Among the DCM extracts, the maximal toxicity (just 30% larval mortality), which differs from the control at $P=0.05$, demonstrated an extract from culture liquid on M1D. In the remaining extracts, the larvicidal activity was noticeably low, the mortality rate was below 30%. No larvae were affected in the control (Figure 2 B).

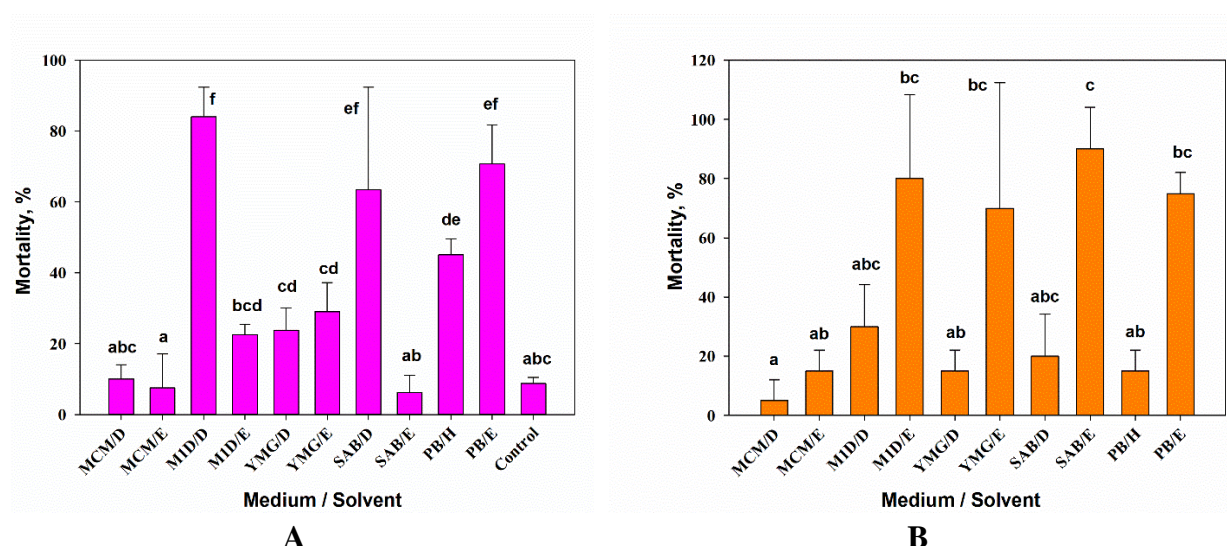


Figure 2. Entomotoxic activity of extracts from *Alternaria tenuissima* MFP253011 cultures on various liquid and solid nutrient substrates: **A** – contact-intestinal aphicidal activity on *Schizaphis graminum* at a concentration of 5 mg/ml (1 mg/dm²) 24 hours post treatment, **B** – acute contact larvicidal activity on *Galleria mellonella* at a concentration of 50 µg/larva 7 days post treatment. Means ± std. deviation marked with the same letter do not differ significantly at the level of P=0.05 according to Tukey's HSD test. D – dichloromethane, E – ethyl acetate, H – hexane.

3.2.2. Phytotoxic activity

All the evaluated extracts of *A. tenuissima* were toxic to leaf segments of both wheat and perennial sowthistle. At the same time, the medium composition combined with the extraction solvent had a significant (P=0.001) effect on phytotoxic activity of the fungal extracts for wheat (H_{9,50}=49.1) and sowthistle (H_{9,50}=45.9) segments according to Kruskal-Wallis one way ANOVA on ranks. EtOAc extracts from the filtrate of *A. tenuissima* cultures obtained on M1D, YMG and SAB media showed relatively high activity on the leaf segments of both plants.

Leaf discs of sowthistle were highly sensitive (necrosis diameter 7–9 mm) to EtOAc extracts from *A. tenuissima* cultures grown on most of the used substrates, with the exception of MCM. DCM extracts from culture liquid and hexane extract from solid culture were generally less toxic: the diameter of necrosis was in the range of 3–5 mm (Figure 3 A). EtOAc extract from the filtrate of culture on YMG was significantly more toxic than DCM extract from the culture liquid produced on M1D medium (P=0.002), as well as than DCM (P=0.03) and EtOAc extracts of from cultures on MCM (P=0.021).

There were significant differences in sensitivity of wheat leaf segments to highly toxic EtOAc extract from the M1D liquid culture of *A. tenuissima* and three non-polar extracts: DCM extracts from MCM (P=0.001) and YMG (P=0.010) liquid cultures as well as hexane extract from PB solid culture (P=0.030) (Figure 3 B).

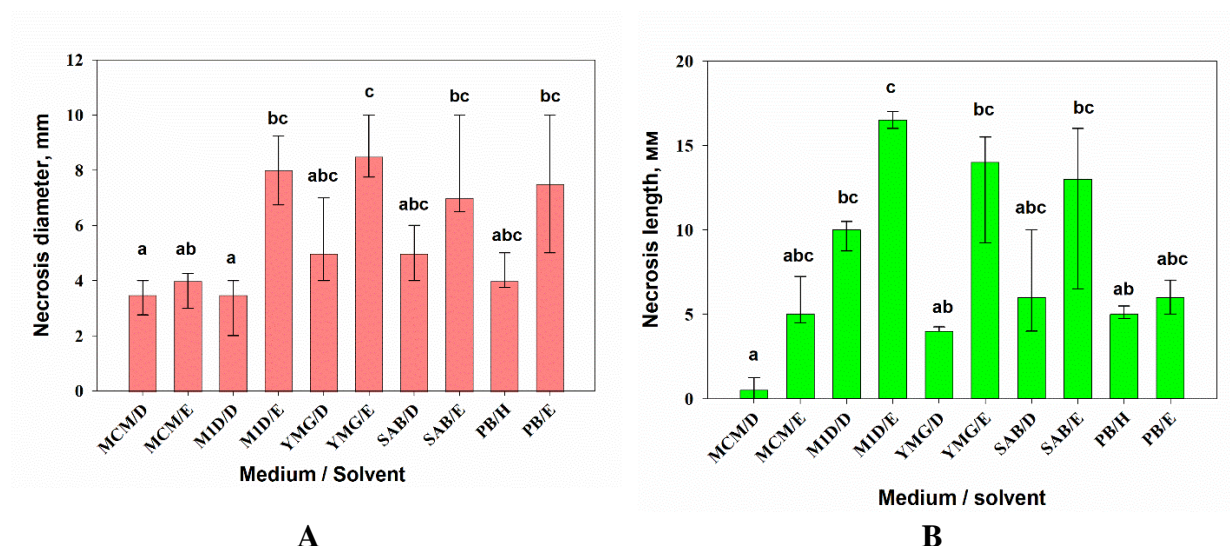


Figure 3. Phytotoxic activity of 0.5% extracts from *Alternaria tenuissima* MFP253011 cultures grown on various liquid and solid media 48 hours post treatment assayed on leaf discs of *Sonchus arvensis* (**A**) and leaf segments of *Triticum aestivum* (**B**). Median values with 25–75% quartiles marked with the same letter do not differ significantly at the level of $P=0.05$ according to Dunn's test. D – dichloromethane, E – ethyl acetate, H – hexane.

3.2.3. Cytotoxic activity

Combined the medium composition and the extraction method had a significant effect ($H_{10,33}=41.9$, $P=0.001$) on the cytotoxicity of 0.01% extracts from *A. tenuissima* cultures according to Kruskal-Wallis one way ANOVA on ranks. DCM extracts from the filtrate obtained from MCM and MID liquid cultures, as well as hexane and EtOAc extracts from the solid (PB) culture of *A. tenuissima* caused 100% mortality of Sf9 cells. The cytotoxicity of DCM extract from the YMG liquid culture was significantly lower ($P=0.014$) than the toxicity of the mentioned extracts. EtOAc extracts from the filtrate obtained from MCM and MID liquid cultures, and DCM extract from the filtrate of fungal liquid cultures grown on Sabouraud's media were, respectively, medium toxic (50–60% cell mortality). EtOAc extracts from the filtrate of fungal liquid cultures grown on semi-synthetic YMG and SAB media were weak (up to 20% cell mortality) toxic (Figure 4).

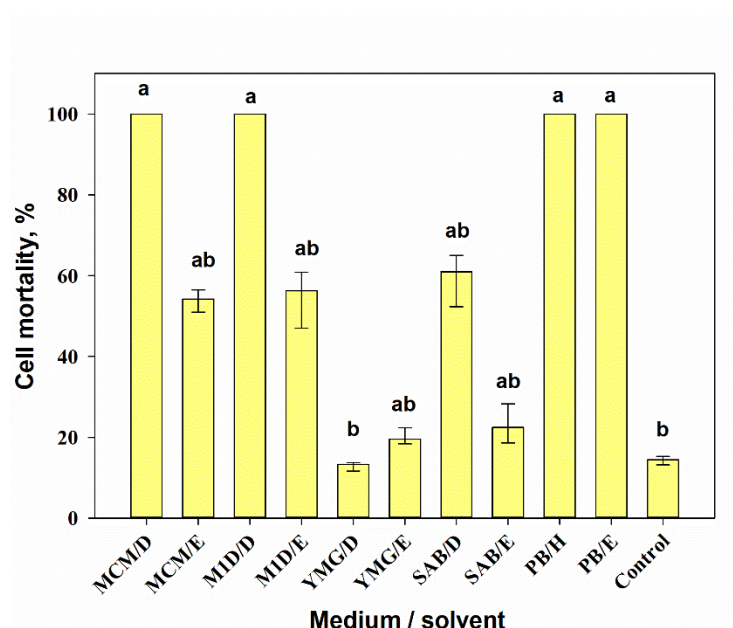


Figure 4. Cytotoxic activity of 0.01% extracts from *Alternaria tenuissima* MFP253011 cultures grown on various liquid and solid media 24 hours post treatment assayed Sf9 cells. Median values with 25–75% quartiles marked with the same letter do not differ significantly at the level of $P=0.05$ according to Dunn's test. D – dichloromethane, E – ethyl acetate, H – hexane.

3.2.4. Antimicrobial activity

The medium composition and the extraction method had a significant effect (one-way ANOVA, $F_{9,20}=107.6$, $P=0.001$) on the antibacterial activity of *A. tenuissima* extracts against the gram-positive bacterium *Bacillus subtilis*. The highest inhibitory activity (10–12 mm lysis zone) was shown by EtOAc extracts (500 µg/disc) from *A. tenuissima* cultures obtained on M1D and YMG media, as well as on the solid substrate. The bacterium was also moderately sensitive to DCM extracts from the culture fluid of the fungus on M1D and YMG media (the radius of the lysis zone is 7–9 mm) (Figure 5). These two extracts considerably suppressed the growth of *C. albicans* (9–11 mm lysis zone), while the activity of others was insignificant (data not shown).

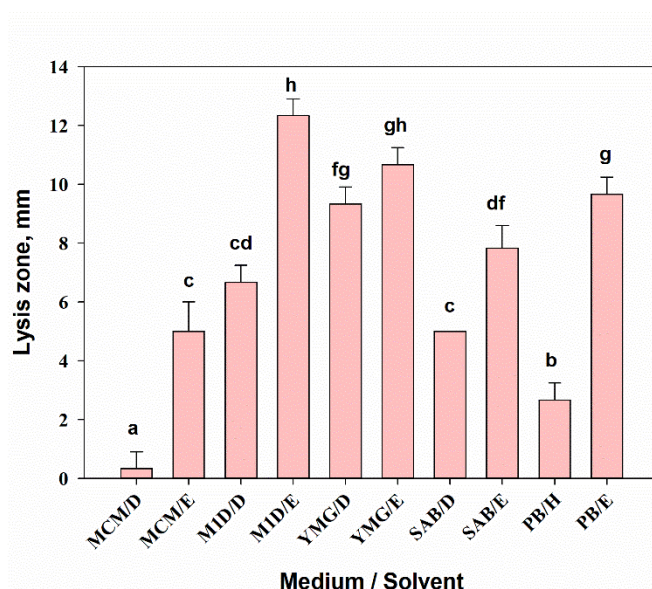


Figure 5. Antibacterial activity of extracts from *Alternaria tenuissima* MFP253011 cultures grown on various liquid and solid media 24 hours post treatment against *Bacillus subtilis*. Means \pm std. deviation marked with the same letter do not differ significantly at the level of $P=0.05$ according to Tukey's HSD test. D – dichloromethane, E – ethyl acetate, H – hexane.

3.3. Analysis of extracts

In the extracts from culture liquid of *A. tenuissima* MFP253011, three known mycotoxins (tentoxin, dihydrotentoxin, tenuazonic acid) were detected by characteristic MS- and UV-spectra. Another group of substances is presumably belonged to the meroterpenoid group, such as ACTG toxins and tricycloalternarenes (chromatography peaks with m/z 321, 345, 349, 363, 377 and with a typical 266–272 nm single UV-absorption band). Their exact identification in the extracts is difficult due to the presence of isomers [28–32].

Three tricycloalternarene (TCA) compounds with mw 348 Da (presumably, isomers of TCA 1) and mw 362 Da (presumably, TCA 11 a/b), as well as a polypeptide (presumably, iso-tentoxin with mw 414 Da) were identified as major metabolites in DCM extract from fungal culture on M1D liquid medium (Figure 6 A), which showed aphicidal and other types of activity (Figure 2 A).

When *A. tenuissima* was cultured on semi-synthetic YMG and SAB media, tentoxin and dihydrotentoxin prevailed in DCM extracts from the culture liquid. The latter extract was more complex and also contained two TCAs (presumably, TCA 4 a/b, TCA 5a/b) with mw 344 Da (Figure 6 B) and showed aphicidal activity (Figure 2 A).

In the EtOAc extracts of *A. tenuissima*, which were highly toxic to *G. mellonella* larvae (Figure 2 B), the major metabolite was tenuazonic acid. Its relative content was high in the extracts from the filtrate of cultures on M1D, YMG and SAB media, while the EtOAc extract from the solid culture had a relatively lower concentration of the toxin due to the presence of other compounds like two substances with mw 320 Da (presumably, TCA A isomers) (Figure 6 C, D).

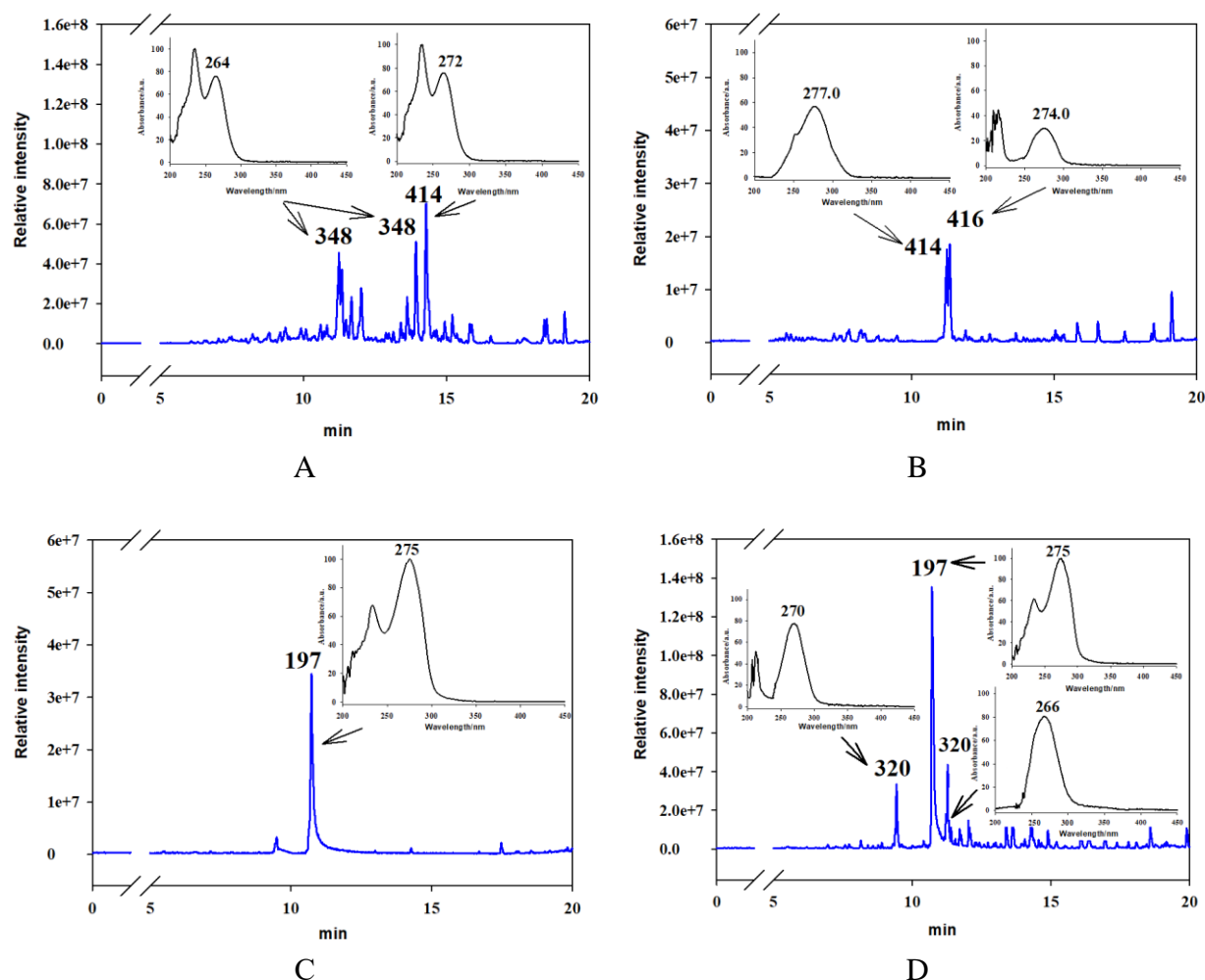
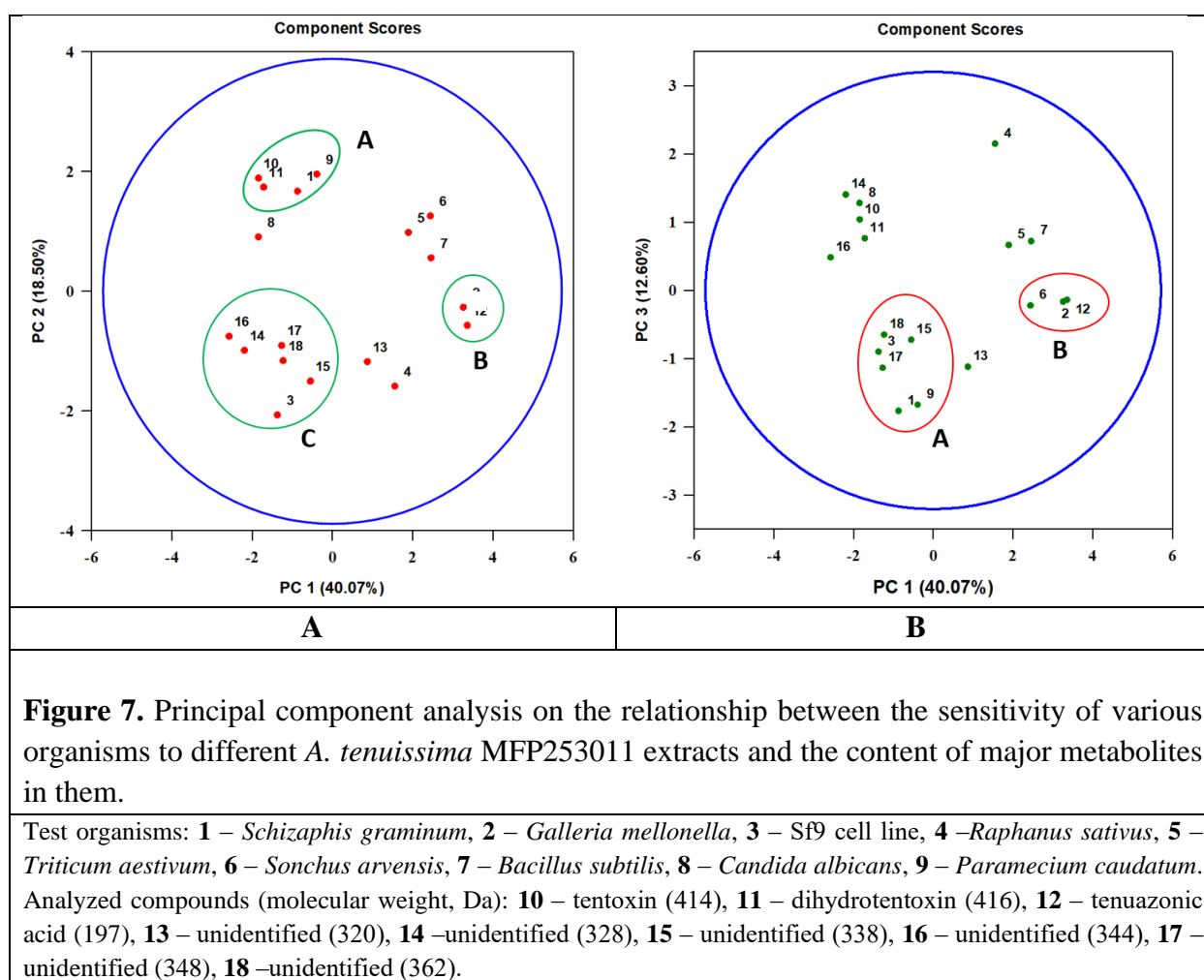


Figure 6. Representative HPLC/MS chromatograms of extracts showing entomotoxic activity from *Alternaria tenuissima* 253-011 cultures on various media obtained with methylene chloride (A-M1D medium, B-Saburo medium) and EtOAc (C – DMG medium, D – pearl barley).

Data on biological activity of 10 extracts on 9 test-organisms and relative content of 9 major metabolites in them were evaluated with PCA. Three principal components (PC) were revealed, which explained 40.1, 18.5 and 12.6% of the data variance, respectively; the effect of the remaining seven components to explain the data was insignificant.

Analyzing PC1 and PC2, it was revealed that the sensitivity of the common wheat aphid, *Schizaphis graminum* and the infusoria, *P. caudatum* to some extracts may be associated with the content of tentoxin and dihydrotentoxin (see cluster A on Figure 7 A). In PC1 and PC3 coordinates, the aphicidal and cytotoxic activity of the extracts seems to be associated with the presence of the metabolites with molecular weights of 338, 348 and 362 Da (see cluster A on Figure 7 B). The sensitivity of *G. mellonella* larvae to extracts was closely correlated with the content of tenuazonic acid (TeA) (see cluster B on Figure 7 A, B).



Sensitivity of Sf9 cells looks to be correlated with TCAs content in the extracts (cluster C on Figure 7A, cluster A on Figure 7B).

Considering the relatively high yield of EtOAc extracts (Figure 1), TeA was found to be the main and easily available exo-metabolite in the liquid cultures of *A. tenuissima* MFP253011 grown on M1D and YMG media. Because these extracts demonstrated relatively high toxicity to *G. mellonella* larvae, TeA was purified from them to confirm the entomotoxic activity.

3.4. Entomotoxic activity of tenuazonic acid

3.4.1. Acute-contact larvicidal toxicity

G. mellonella larvae were sensitive to the injection of TeA in the tested concentrations. The dead caterpillars became melanized. A statistically significant difference between the survival curves was revealed (log-rank test: $\chi^2=15.5$, $df=4$, $p=0.004$). At concentrations of 20 $\mu\text{g/larva}$ ($LT_{50}=6\pm1.3$ days) and 50 $\mu\text{g/larva}$ ($LT_{50}=6\pm0.8$ days), TeA significantly (at $P=0.05$) decreased survival rate of *G. mellonella* larvae compared to control (Figure 8 A).

Zophobas morio larvae were also sensitive to injection of various concentrations of TeA (Figure 8 B). There was a statistically significant difference between the survival curves (log-rank test: $\chi^2=12.7$, $df=3$, $P=0.005$). At concentrations of 20 $\mu\text{g/larva}$ ($LT_{50}=4\pm4.5$ days) and 50 $\mu\text{g/larva}$

($LT_{50} = 2 \pm 0.6$ days), TeA significantly (at $P=0.05$) decreased survival rate of *Z. morio* larvae compared to control.

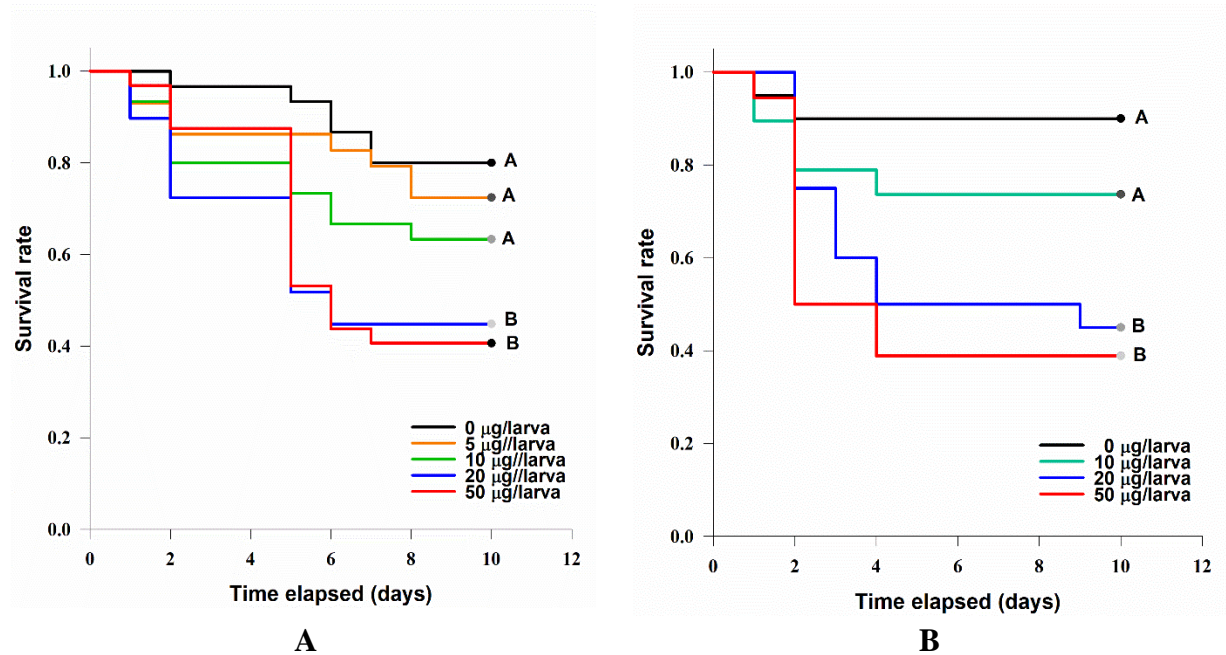


Figure 8. Survival curves of *Galleria mellonella* (A) and *Zophobas morio* (B) larvae after injection of various concentrations of tenuazonic acid. The curves marked with one letter do not differ significantly at $P=0.05$ by log-rank test with Holm-Sidak adjustment.

3.4.2. Oral toxicity

Two-way ANOVA of the lg-transformed data showed that TeA concentration in the artificial feed strongly ($df\ 4$, $F=70.1$, $P<0.001$) affected the biomass of *G. mellonella* larvae, while the time factor was insignificant ($df\ 5$, $F=1.4$, $P=0.25$). The interaction of the factors, "time" and "TeA concentration", was significant ($df\ 20$, $F=6.4$, $P<0.001$) meaning existing of different trends. A significant (at $P=0.05$) decrease of larvae biomass accumulation compared with the control was noted at TeA concentration of 2.5 mg/g of the feed, starting from the 4th day post treatment; by the 10th day of observations, it became about 2 times lower than in the control. At a concentration of 0.25 mg/g of feed, inhibitory effect of TeA was pronounced on the 6th day of the experiment (Figure 9). Visually, *G. mellonella* larvae refused to feed containing TeA. This probably explains why their mortality did not exceed the level of 20% by the 10th day of observations at a TeA concentration of 25 mg/g of feed.

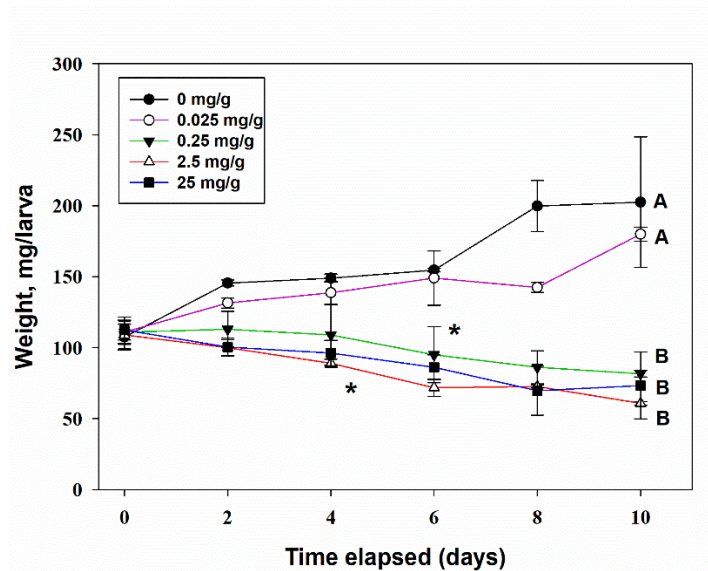


Figure 9. The effect of the concentration of tenuazonic acid in artificial feed on the growth of the biomass of *Galleria mellonella* larvae. Means \pm std. deviation marked with the same letter do not differ significantly at the level of $P=0.05$ according to Tukey's HSD test. * – significant difference from control at $P=0.05$ by Dunnett's test.

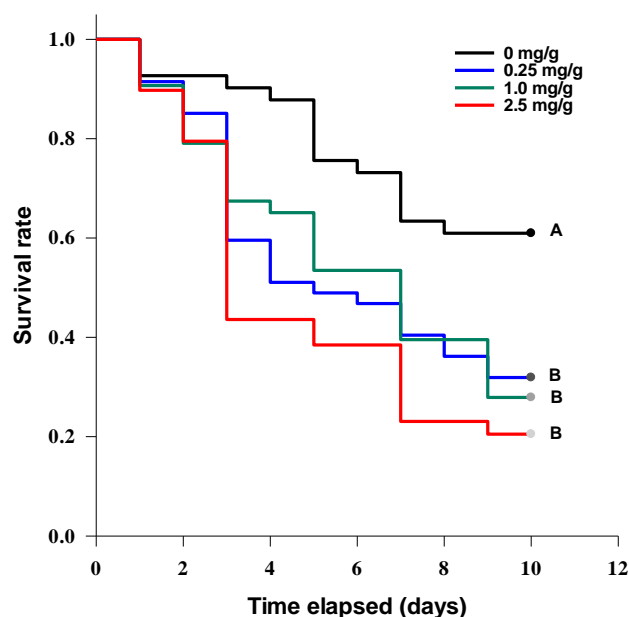


Figure 10. The survival curves of *Acheta domestica* imagines when adding different concentrations of tenuazonic acid to the feed. The curves marked with one letter do not differ significantly at $P=0.05$ by log-rank test with Holm-Sidak adjustment.

After 2 days of incubation, *Acheta domestica* imagines completely consumed the toxin-spiked and control feed, and in all the treatments it was replaced with fresh (without the addition of TeA). A statistically significant difference (log-rank test: $\chi^2=16.4$, df 3, $P=0.001$) between the survival curves of the crickets fed with different TeA content was revealed (Figure 10). The cricket mortality rate was significantly (at $P=0.05$) higher than the control at all TeA concentrations used, but the differences between the treatment options were insignificant. The median survival of the

cricket was 3 ± 0.2 , 5 ± 1.5 and 7 ± 0.9 days at the toxin concentrations of 2.5, 1 and 0.25 mg/g of feed, respectively.

3.4.3. Contact-intestinal activity

The concentration of TeA had a significant (one-way ANOVA, $p=0.001$) effect on the mortality of the common wheat aphid ($F_{3,8}=28.4$) and female of spider mites ($F_{3,16}=31.4$) 24 hours after treatment. Significant (at $P=0.05$) aphicidal activity of TeA was noted at a toxin concentration of 1.0 mg/mL (27% mortality), the acaricidal action was revealed at its concentration of 0.5 mg/mL (12% mortality). At a maximal concentration of 2 mg/mL, the mortality reached 43% and 19% for the aphids and mites, respectively (Figure 11 A, B). Beauvericin caused 100% mortality of the tested arthropods at a concentration of 0.5 mg/mL.

No symptoms of phytotoxicity were manifested on wheat leaf segments soaked in TeA solution, whereas small necrosis was noted on bean leaf cuttings at a TeA concentration of 2 mg/mL 24 hours after treatment.

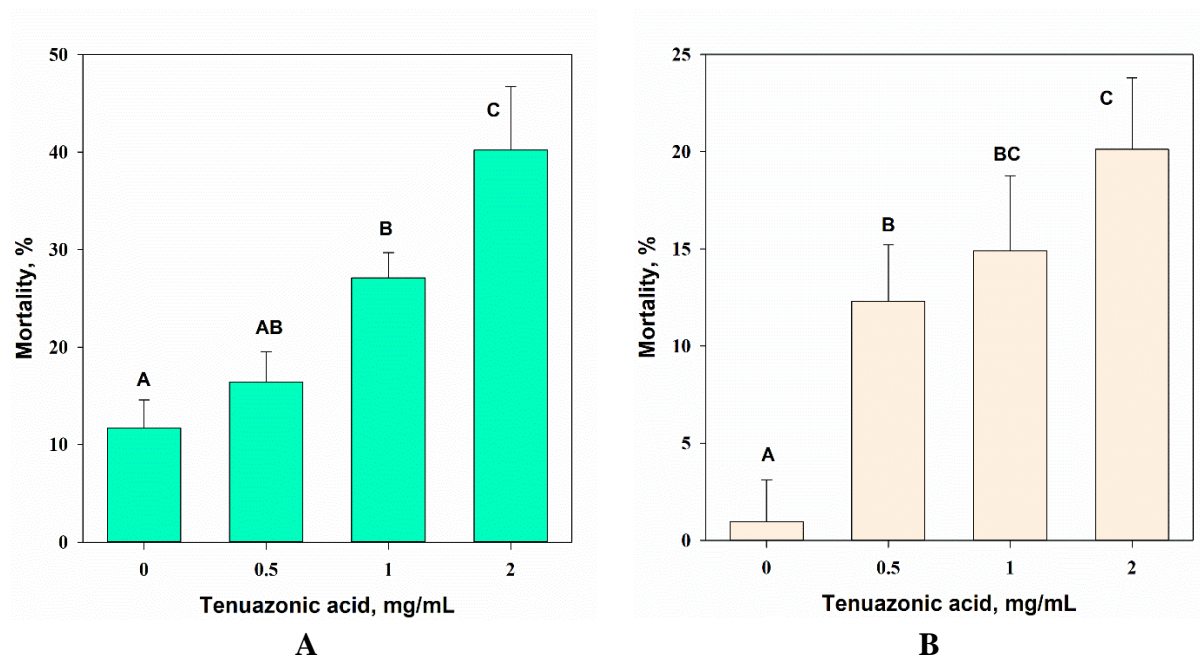


Figure 11. Toxicity of various concentrations of tenuazonic acid against *Schizaphis graminum* (A) and *Tetranychus urticae* (B) 24 h post treatment. Means \pm std. deviation marked with the same letter do not differ significantly at the level of $P=0.05$ according to Tukey's HSD test.

3.4.4. Cytotoxic activity

TeA was about 5 times less toxic with IC_{50} 25 μ g/mL (126 μ M) than beauvericin with IC_{50} 4 μ g/mL (5 μ M) against Sf9 *Spodoptera frugiperda* cells. Unlike beauvericin, at all the studied concentrations, TeA caused fast degradation of Sf9 cells. It should be noted that the exact calculation of the IC_{50} for TeA was difficult due to the fact that the dead cells were rapidly destroyed (Figure 12).

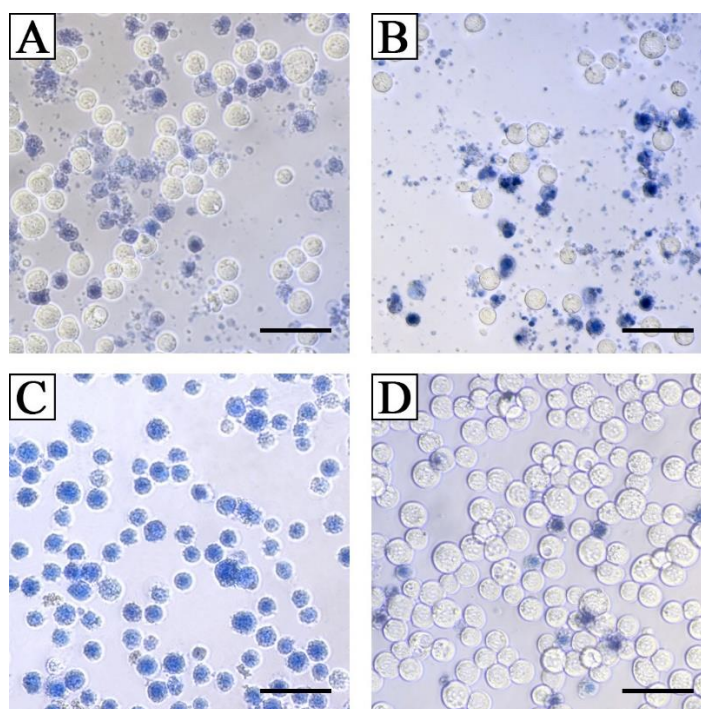


Figure 12. Morphology of Sf9 cells after 24 h of exposure with tenuazonic acid: (A - 15 µg/mL, B - 25 µg/mL) and beauvericin (C - 25 µg/mL) on Sf9 cells; D - control. Bar indicates 50 µm.

4. Discussion

4.1. Detection of entomotoxic metabolites in *A. tenuissima* extracts

Metabolites of small-spored *Alternaria* species have been characterized as mycotoxins (tenuazonic acid, alternariol and its methyl ester, altertoxins) and as substances with phytotoxic (e.g. altenuene, tenuazonic acid, tentoxin and a number of host-specific toxins), antimicrobial (e.g. altersetin), cytotoxic activity (bi - and tricycloalternarenes) and other interesting properties (enzyme inhibitors, antioxidants, etc.). Many of them possesses several types of biological activity [33–35]. In the present and earlier studies [21], the extracts from *A. tenuissima* MFP253011 cultures obtained by various methods (using different media and solvents) were found to display entomotoxic activity along with phytotoxic, antimicrobial and cytotoxic properties (Figures 2-5). A number of known (tenuazonic acid, tentoxin, dihydrotentoxin) and unidentified compounds belonging to the tricycloalternarene group were detected, but their composition varied depending on the production method (Figure 6). This allowed us to evaluate the relationship of the entomotoxic activity of *A. tenuissima* extracts with other bioactivity types together with the formation of nine major metabolites (Figure 7). A similar approach was successfully implemented to identify the determinants of the pathogenicity of *Ilyonectria mors-panacis* affecting ginseng [36] and the biological activity of the aquatic micromycetes, *Aspergillus awamori* [37].

Analysis of the composition of extracts and their activity by PCA revealed the aphicidal activity of extracts to be associated with the presence of tentoxin and dihydrotentoxin, as well as some tricycloalternarenes (TCA), for example, TCA 1 a/b (mw 348 Da), TCA 11 a/b (mw 362 Da) and others (Figure 7). However, entomotoxic activity of the compounds is unknown while their cytotoxic activity has not been sufficiently studied. For instance, weak cytotoxicity of tentoxin against HepG2 [38] and HeLa cell lines [39] as well as some TCAs for various tumor cell lines [40,41] was reported. Relation between the production of TCAs and cytotoxic activity of *A.*

tenuissima MFP253011 extracts against Sf9 cells is more likely (Figure 7). Phytotoxicity of tentoxin [42] and TCA 1 a/b [43] to wheat, the host plant of *S. graminum*, may be responsible for indirect effect of these substances on the aphid. In general, the spectrum of biological activity of tentoxin, dihydrotentoxin and TCAs is still poorly understood [44], and an evaluation of their anti-insectan properties may be interesting. However, the yield of extracts containing these metabolites was relatively low (up to 200 mg/L) (Figure 1). In this regard, this work may be planned for the future with optimized media conditions for their production.

PCA indicated tenuazonic acid (TeA) to be responsible for the larvicidal toxicity of some EtOAc extracts against *G. mellonella* (Figure 7), a model insect for studying the effects of toxins, immunosuppressants, antibiotics, as well as the relationship between pathogens and insects [45]. According to the literature, TeA was entomotoxic to the first instar larvae of the green bottle fly *Lucilia sericata* (LD₅₀ 120 µg/ml), but was not active to other tested arthropods from various orders: *Drosophila melanogaster* (Diptera), *Sitophilus granarius* (Coleoptera), *Aphis fabae* (Hemiptera) and *Tetranychus urticae* (Trombidiformes) [11]. There are no data on the sensitivity of *G. mellonella* to this toxin. In order to study entomotoxic properties TeA, it was isolated from the culture of *A. tenuissima* MFP253011.

In liquid cultures of various strains of small-spored *Alternaria* spp., TeA content was detected with a yield of about 20-40 mg/L. Commonly, the producer strains were grown on both mineral (Richard's and Czapek) and semi-synthetic (potato-dextrose broth, YES and Richard's + 0.5% peptone) liquid media. The last medium allowed to reach maximal toxin yields of up to 100–300 mg/L. When the fungi were grown on solid substrates, the yield of TeA reaches 200 mg/kg [46–50]. TeA was found to be a major toxin (up to 79% of the total content) in extracts of some *A. tenuissima* strains [51]. In our study, a relatively high yield of TeA was obtained (105 mg/L, ~17% of the extract) by submerged fermentation of *A. tenuissima* MFP253011 in a bioreactor on YMG medium and by using the standard procedures for the toxin isolation from the culture liquid. This may indicate TeA as an easily available metabolite of *A. tenuissima*, which can be used for various biological assays requiring large amounts of the substances like entomotoxicity tests.

4.2. Entomotoxicity of tenuazonic acid

The injection method using *G. mellonella* larvae has been successfully tested for the toxicological characterization of various chemicals [23]. For example, okadaic acid from shellfish at concentrations ≥ 75 ng/larva led to a significant decrease in the survival rate of *G. mellonella* (> 65% mortality) 24 hours after injection with LD₅₀ ~ 239 µg/kg comparable to its toxic dosage for rats [52]. This method has been used to assess the acute toxicity of metabolites of entomopathogenic fungi. In particular, a purified mixture of efraeptins from *Tolypocladium* spp. was toxic to *G. mellonella* with an LD₅₀ 30 ng/larva [53]. At a dosage of 8.6 µg/larva, beauvericin caused 37% mortality of *G. mellonella* after 12 days incubation with the median lethal time (LT₅₀) about 6 days [54]. LD₅₀ of cordycepin was about 200 µg/larva 6 days post injection [55]. In our experiments, a significant decrease in *G. mellonella* viability was observed at a concentration of 20 µg/larva (ca. 100 mg/kg) with LT₅₀ ~ 7 days (Figure 8A). It was also shown for the first time that *Zophobas morio* larvae, a food insect used, for example, for feeding chickens [56], were also sensitive to injection of TeA at a dosage of 20 µg/larva (~ 140 mg/kg) with LT₅₀ ~ 4 days (Figure 8B). These data demonstrate moderate level of acute larvicidal activity of TeA comparable with entomotoxicity of beauvericin and cordycepin.

In oral assays, addition of inactivated *Fusarium* spp. cultures to the feed for *G. mellonella* allowed to identify the toxin-producing strains [57]. Diacetoxiscirpenol and neosolaniol isolated from *F. sambucinum* reduced the consumption of the spiked feed by *G. mellonella* ca. 50% compared to untreated control on caterpillars at a concentration of 50 µg/g of feed [58]. Oosporein from *Beauveria brogniartii* was nontoxic for the insect at a concentration of 142 µg/g of feed [59]. Bioactive alkaloids, harman and nonharman, found in the entomopathogenic fungus *Conidiobolus coronatus*, in a non-lethal concentration of 1.25 mg/g of feed caused a significant delay in the development of *G. mellonella* [60]. In our experiments, no dead *G. mellonella* larva was observed at TeA concentration of 2.5 mg/g of feed within 10 days of incubation. Moreover, TeA was not 100% lethal even at 25 mg/g (data not shown). However, at a concentration of 250 µg/g of feed, the growth of larval biomass was significantly inhibited (Figure 9). Low toxicity coupled with biomass loss may indicate rather antifeedant effect of TeA for *G. mellonella*; however, this hypothesis needs further evidence. Nothing has been known about the effect of mycotoxins on another food insect, *Acheta domesticus* [61]. When TeA was added to the cricket diet, a significant insect mortality was observed at a concentration of 250 µg/g of feed with LT₅₀ for about 7 days (Figure 10).

In the contact-intestinal assays, TeA was found to have moderate aphicidal activity with 40% mortality 24 hours after treatment at a concentration of 2 mg/mL (Figure 11A), whereas beauvericin was considerably more toxic causing 100% mortality at a lower concentration of 0.5 mg/mL. However, according to [62], beauvericin at the same concentration was less toxic to *S. graminum* (23% mortality). The differences in aphicidal activity of beauvericin may be explained by variability of *S. graminum* populations. As mentioned above, some other fungal toxins of the genus, for example, diversolonic esters and chloromonilinic acid B, are more toxic than TeA causing 70–80% mortality of *S. graminum* at a concentration of 1 mg/mL [9,63]. The insecticide thiacloprid from the group of neonicotinoids was significantly more toxic to grass aphids with an LD₅₀ of about 210 µg/mL [64]. The sensitivity of aphids to seven organophosphorus insecticides varied within the LD₅₀ range from 0.1 to 60 µg/mL, depending on the level of resistance of *S. graminum* populations [65].

T. urticae turned out to be less sensitive to TeA than *S. graminum*. However, significant mortality slightly more 10% was detected at 0.5 mg/mL after 24 hours of exposure (Figure 11). Beauvericin showed noticeable acaricidal properties with LD₅₀ from 0.66 to 33 µg/mL, depending on the sensitivity of the *T. urticae* population to the toxin [66]. The extract of the soil micromycete *Aspergillus melleus*, in which mellamide, ochratoxin C, nodulisporic acid, 7-oxocurvularin, and 6-(4'-hydroxy-2'-methyl phenoxy)-(-)-(3R)-mellein were detected, was significantly less toxic to the mites with LD₅₀ 10 mg/L [67]. Thus, TeA demonstrated low activity against sucking insects in the contact-intestinal assays. From other hand, evaluation of antifeedant and ovicidal effects of TeA on *S. graminum* and *T. urticae* looks interesting for a further study.

Testing entomotoxic substances on insect and mammalian cells is helpful for the primary determination of the mechanisms of their action on arthropods [68]. Among 65 mycotoxins tested, TeA had relatively weak cytotoxicity to both insect and mammalian cells. It was shown that Sf9 cells are two times more sensitive to beauvericin than to TeA [69]. When tested on the porcine intestinal columnar epithelial cells (IPEC-J2 line), the cytotoxicity of TeA was 10 times lower than that of beauvericin [70]. However, in contrast to the latter, TeA caused rapid destruction of Sf9 cells at the minimum studied concentration of 15 µg/mL (Figure 12) and, therefore, may pose an interest for mode of action studies.

4.3. Tenuazonic Acid as a Model Molecule

TeA, a derivative of tetramic acid, may be of particular interest as a model molecule since: 1) it is produced by a number of phytopathogenic fungi (*Alternaria* spp., *Magnaporthe oryzae*, *Phoma sorghina*) [71], 2) this toxin is one of the most common pollutants of food products, while its toxicity to invertebrates and vertebrates has been poorly understood [72,73], 3) there are acaricides and insecticides (acetyl-CoA carboxylase inhibitors) from the tetramic acid group (for example, spiropidion) that are effective against sucking arthropods [74]; 4) TeA, along with some other tetramic acid derivatives, is a promising natural herbicide [75], whereas its toxicity to beneficial insects (for example, pollinators, entomophages and feed protein producers) has not been studied; 5) methods of chemical synthesis of TeA and its derivatives with various useful properties have been developed [11, 71, 76, 77].

TeA is a fungal metabolite that is found with a high frequency in food and feed. Commonly, TeA concentration in the natural material does not exceed the level of 100 ng/g and obviously is not entomotoxic. However, the maximum TeA content in food may vary greatly, reaching relatively high concentrations: up to 0.7 µg/g in grain products, up to 8 µg/g in vegetables, and up to 20 µg/g in spices [78,79]. However, in cases of strongly moldy substrates or of provisional using TeA as an herbicide, the maximal concentrations can be sharply exceeded making both harmful and useful insects (pollinators, entomophages, protein producers, etc.) sensitive to it.

TeA has several molecular targets in the cells of various organisms. In plant cells, it inhibits the proton pump of the plasma membrane and photosynthesis [80,81], being one of the pathogenicity factors of phytopathogenic fungi producing it [82]. Cell-free assays demonstrated TeA to be an antioxidant and a promising inhibitor of acetylcholinesterase and β-amyloid aggregation [83,84]. TeA is toxic to rodents and chickens, causing diarrhea, bleeding and precancerous conditions. Probably, the toxin negatively affects the digestive system of sensitive animals, but the exact mechanism of action of TeA is still unknown [73,85].

The most well-known mechanisms of action of insecticidal and acaricidal compounds are malfunction of nervous system, blocking of postsynaptic receptors, inhibition of respiration, and developmental disorders [86]. The absence of considerable behavior and developmental changes, the lack of tremor, the weak sensitivity of sucking phytophages as well as an obvious antifeedant effect may indicate TeA is not an inhibitor of lipid biosynthesis like spirotetramat, but the intestinal toxin for sensitive insects [86]. From other hand, decreased viability and fertility of arthropods can also be achieved by the indirect action of TeA – by suppressing their symbiotic microflora and humoral immunity, as well as deterioration in the feed quality of the substrate [1]. At high concentrations (~1 mg/mL), TeA is a phytotoxin, killing sensitive plant cells, at lower concentrations it can elicit immune reactions in plants [87,88]. Reduced the level of nutrition can weaken herbivorous arthropods to increase their susceptibility to various biotic (e.g. increased susceptibility to pathogens) and abiotic adverse factors.

5. Conclusion

TeA, being a main metabolite of *Alternaria tenuissima* and some other phytopathogenic fungi, displays moderate entomotoxic properties, which are manifested in injection and oral tests. TeA seems to be not only a virulence factor but also to play a role in a competition of the phytopathogenic fungi with herbivory arthropods. The mode of action of this toxin can be evaluated further using insect model systems, for example, on *Galleria mellonella* larvae and Sf9

cells serving by the way to solve partly the enigma of its mammal toxicity. Another interesting issue of future studies is to determine the indirect effects of TeA on arthropods: is the entomotoxic effects correlated with phytotoxicity, is the intestinal microflora of arthropods suppressed, does this toxin affect their humoral immunity and sensitivity to entomopathogens?

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