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Research Article

Trichothecenes Production by Entomopathogenic Fungus *Conidiobolus Coronatus*

Abstract

Entomopathogenic fungi are of particular interest now as the likely source of a new class of insecticides. This interest stems from the fact that these organisms naturally present in the environment, in most cases selectively affect specific organisms, and metabolites produced by them do not pass into the food chain. Using naturally or artificially introduced organisms to reduce populations of arthropod pests, provide you more than ever the security of the consumers as well as crops and the environment. This is particularly important from the point of view of organic farming, in which there is a need to extend the scope of alternatives against harmful insects. Careful study of the selection of organisms used, as well as the methods of their application are necessary for the efficient and safe prevent losses caused by the pests. Much attention is paid to these issues, with a view to both human health and reducing exposure to unintended infections other living organisms, non-target conservation measures. The aim of this study was to identify toxic metabolites from trichothecene group produced by the parasitic fungus *Conidiobolus coronatus*. The results of our study allow conclude that the T-2 toxin is present in *C. coronatus* extract. It was also shown that *C. coronatus* produced higher amount of trichothecenes when is grown on LB medium. The optimal temperature for production of both T-2 and HT-2 by *C. coronatus* was 20°C. However, the pH value is the same for grown of *C. coronatus* mycelium and trichothecenes production. In any of the tested extracts DON and NIV was not detected. Toxicity of *C. coronatus* homogenates and post-incubation filtrates to *G. mellonela* larvae and Sf9 cells was also determined.

Introduction

Entomopathogenic fungi are a particularly interesting source of new classes of bioinsecticides. Due to the necessity to reduce the amount of chemical insecticides entomopathogenic fungi are becoming more and more popular as bio-insecticides [1]. The use of naturally or artificially introduced organisms to reduce populations of arthropod, provide more than ever future consumer safety, as well as plants and the environment. This is especially important for organic farming in which there is no need to extend the range of alternatives to harmful insects [1,2]. Hitherto, the use of insecticidal fungi in crop as well as the forest protection has been limited. Currently, more than 100 species of fungi is the subject of research focused on their use to reduce the number of arthropod pests [3]. Careful study of the selection of fungus as well as the methods of their use in the environment are necessary for the efficient and safe the prevention of losses caused by the pests. Practical application as a source of bioinsecticides have mainly fungi belonging to Type fungi Imperfecti [1]. The most common and most widely studied species include: *Beauveria bassiana*, *Beauveria brongniartii*, *Metarrhizum anisopliae*, *Paecilomyces fumosoroseus* and *Verticillium lecanii* [2]. Nowadays more and more attention as a potential source of bio-insecticides has an enthomopathogenic fungus *Conidiobolus coronatus*. Research on the use of the insecticidal potential of this fungus in forestry and agriculture are still running. *C. coronatus* is an opportunistic pathogen of fairly broad range of infected hosts. This entomopathogenic fungus causes the disease process in many arthropods eg. *Galleria mellonella*, *Dendrolimus pini*, and others [1,5-8]. *C. coronatus* causes rapid death of the insects, perhaps by the secretion of toxic metabolites [9-12]. Insects infected by this parasite die within 1-2 days. An infections caused by *C. coronatus* resulted in the appearance of black spots on the body surface larvae. As a result, dead insect's cuticle becomes completely black. Inside the infected host hemocel not observed structures of the fungus, suggesting that the death of the larvae is caused by mycotoxins produced by *C. coronatus* and not by exhaustion of nutrients. In insects that survived to infection with *C. coronatus*, morphological changes of Malpighiego coils were observed. They become dark and brittle. The conversion are caused by detoxification and elimination of toxic metabolites

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secreted by parasitic fungus [12]. Because of insecticidal potential, a relatively easy laboratory culture *C. coronatus* can be used as a source of new generation bioinsecticides. Trichothecenes are a family of closely related sesquiterpenoids and subdivided into four types (A-D) according to structural similarities. [13]. Most of them have a double bound at position C-9,10, a 12,13 epoxide ring and a variable number of hydroxyl and acetoxy groups. Trichothecenes are produced by *Fusarium, Myrothecium, Trichothecium and Cephalosporium sp.* The type-A and -B trichothecenes usually are found in grain and are therefore often contaminants of food and feed. Type-A trichothecenes have an ester bond at C-8, while the type-B trichothecenes possess a ketone group at C-8. In cereal grains, T-2 toxin and HT-2 toxin are regarded as the most important type-A trichothecenes, while deoxynivalenol (DON), and its 3-acetyl-derivative (3AcDON) as well as nivalenol (NIV) are regarded as the most important type-B trichothecenes. These trichothecenes possess a potent cytotoxicity to eukaryotic cells, and this biological activity is closely related to their lethal and dermal toxicity, cellular damage to actively dividing cells, impairment of immunoresponses, and inhibition of macromolecule syntheses [13]. Considering the above, the aim of the study was to develop methods for the isolation of the mycotoxins from the group of trichothecenes, as well as qualitative analysis of these compounds using chromatographic methods. Moreover, identification and determination of toxic metabolites from trichothecene group in LB and MM media and post-incubation filtrates of *Conidiobolus coronatus* were done. The effect of temperature and pH on production of trichotecene toxin by *C. coronatus* was also checked. Toxicity of *C. coronatus* homogenates and post-incubation filtrates to *G. mellonela* larvae and Sf9 cells was also determined.

Chemicals

T-2 toxin, HT-2 toxin, DON, NIV and 3-AcDON were obtained from Sigma. HPLC-grade acetonitrile was purchased from POCh. Stock solutions were prepared by dissolving in acetonitrile and then further diluted to a final the concentration of 100 mg L^{-1} or less with 50/50 acetonitrile/water prior to mass spectrometric analysis.

Conidiobolus coronatus and post-incubation filtrates

The subject of the studies was the entomopathogenic fungus *C. coronatus* belonging to the class of Zygomycota and the order of Entomophthorales. Mycelium was obtained from cultures of the insecticidal fungus C. coronatus incubated for 3 weeks in 30 liters of liquid medium. The effect of temperatures (20 and 30 ºC), pH (in the range from 5 to 9) and different type of growing media: LB (rich medium), and MM (minimal medium) were checked.

Derivatization of trichothecenes

Trimethylsilylation: A 100 µl of NIV, DEO, T-2, HT-2 stock solutions were taken and placed in vials, then evaporated under a nitrogen stream to dryness. A 100-µl volume of the derivatisation mixture: TMSI or BSTFA + TMCS (99:1) was placed into each vials. The mixture was heated at 60 °C for 30 min. or 80 °C for 60 min. Then the samples were transferred to the inserts and thus prepared trichothecenes derivatives was analyzed by GC-MS.

Acylation: (a) From standard solutions of nivalenol, deoxynivalenol, T-2 toxin and HT-2 toxin a 100 µl were taken and introduced into vials. Then samples were evaporated in a stream of nitrogen to dryness. To dryness residues a 100 µl of acetic anhydride and 1 mg of anhydrous sodium acetate were added, and and heated at 80 °C for 60 min. After the mixtures were cooled and then evaporated three times with toluene in a nitrogen stream (to remove the acetic anhydride residues). Obtained in this way derivatives of trichothecenes were extracted with methylene chloride (4 x 100 µl). Next, the sample was evaporated to a volume of 100 µl under a stream of nitrogen and then transferred to inserts and analyzed by GC-MS.

(b) The dryness residues was trifluoroacetylated with 100 ml of TFAA in the presence of 10 mg of sodium hydrogencarbonate. The mixtures were heated for 30 min at 60 °C in a heater block. After this time mixtures were cooled and the excess of derivatising reagent was evaporated under a gentle stream of nitrogen. Then, 500 µl of toluene and 1 ml of redistilled water were added and this mixture was shaken for 5 min. The organic layers were transferred into vials and concentrated to 100 µl and then analyzed using GC-MS.

GC-MS analysis: Chromatographic analysis was performed using a gas chromatograph coupled with mass spectrometer GC-MS-QP2O1OSE (Shimadzu) equipped with a chromatographic column Rtx-5 (30 mx 0.25 mm x 0.25 microns, Restec). The GC-MS determination was carried out under the following conditions: helium was used as a carrier gas, the temperature of the injection port was 310 °C and the temperature of the ion source (EI) was 200 °C. The column temperature program was: 100 °C held for 1 min, 6 °C/min to 310 °C held for 10 min. A 1-µl volume of the solution was injected.

LC-MS analysis: Chromatographic analysis was performed using liquid chromatography Series 1200 (Agilent Technologies) with Ultra HCT mass spectrometer (Brucker Daltonics), equipped with a Hypersil Gold aQ C18 column (150×4,6 mm, 5 µm). As the mobile phases: ACN (phase), 1 mM aqueous solution CH_3COONH_4 (phase B): ACN (90:10, v / v) at pH 3.5 were used, the separation was carried out under gradient elution conditions from 10% B to 90% phase B (20 min.) and then 90% of phase B by 5 minutes. The parameters of the mass spectrometer was chosen so that the ratio of the analyte signal to noise ratio was the highest: drying gas - nitrogen, drying the gas pressure - 10 psi, flow rate of the drying gas - 7 l/min., temperature of the drying gas - 300 ° C, capillary voltage - 4 kV.

Optimization of extraction conditions

Recovery experiments were carried out on liofilized *C. coronatus* mycelium spiked with different concentration of trichothecenes. To 1 g of finely ground sample 100 µl of a type B trichothecenes mixture in chloroform were spread over the

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surface. The flask was shaken manually to distribute the added standards as evenly as possible. The sample was left open at room temperature for 2 h. Any remaining chloroform was evaporated under a stream of nitrogen. Three extraction solvent mixtures such as: methanol-water (70:30, v /v), acetonitrile -water (85:15, v /v); acetonitrile -water (70:30, v /v) and ethyl acetate-acetonitrile-water (77:19:4, v/v/v) were used. Then samples were placed in a PTFE flasks and mixed with each type of solvents mixture (3 x 15 mL) for 10 min. After filtering through Whatman No. 4 filter paper, the filtrate was defatted with hexane (2x10 ml) and extracted with dichloromethane (3x15 ml). The combined extracts were evaporated to dryness in rotary evaporator. In the same way a sample of *C. coronatus* mycelia without analytes addition was also prepared.

Extraction of trichothecenes from C. coronatus mycelium grown in optimal and stressful conditions

The mycelium obtained after culture of *C. coronatus* obtained in optimal and stressful conditions was liofilized and extracted three times (3 x 15 mL), with a mixture of ACN:H_2O (85:15, v/v). The resulting extracts were filtered through filter paper, and then extracted twice with hexane (2 x 10 mL) to defat them nd extracted with dichloromethane (3x15 ml). The hexane layers were discarded, and the remaining layers were evaporated to dryness using a rotary rotor and then dissolved in 1 ml of ACN. The procedure described above was also used for the extraction of the trichothecenes from post-incubation filtrates.

Determination of the toxicity of the post-incubation filtrates and C. coronatus homogenates obtained in optimal and stressful conditions

***In vivo* tests:** *Galleria mellonella* larvae from 7th larval instar were used as biological model for in vivo toxicity tests. 7th larval instar were injected with 5 µl of tested filtrates, on each test 10 larvae were used. As a control served larvae injected either with IPS, or pure medium, or not injected at all. The observations were held by 21 days till all mature moths were dead.

***In vitro* tests:** 20 samples of fungal mycelium homogenate from *C. coronatus* were cultivated in optimal and stressful conditions. These homogenates have been tested for cytotoxic activity on *G. mellonella's* hemocytes and the continuous insect cell line Sf9 Cell cultures of *G. mellonella's* hemocytes and Sf9 were maintained in vitro in appropriate incubation medium. After suitable preparation of fungal homogenates: reconstitutions, sonification, centrifugation and filtering. Cells were exposed to the homogenates and cytotoxic effect were measured by reading the fluorescence of calcein AM (a cell-permeant dye that determine cell viability) or in Sf9 cell line reading the absorbance in WST-1 test. Tests were carried out in 96 well transparent plates, which contained monolayer of Sf9 cells. To each well diluted homogenates were added with different proteins concentration 100 and 250 µg/ml. After incubation for 48h WST-1 were added and after 3,5h cytotoxicity was determined by reading the absorbance, two wavelength were used 440 and 650 nm. As positive control (high toxic effect on insects cells) neurotoxin - chlorfenvinphos (80) was used, negative control was cells with IPS, or cells without any added. The percentage of dead cells in comparison with the control indicated toxic effect of studied fractions.

Results and Discussion

Optimization of analytical methods for the qualitative and quantitative determination of trichothecenes

Gas chromatography coupled with mass spectrometry is the appropriate for identification and quantification of various compounds in complex matrices. Due to the complexity of biological matrices which are mycelium and post-incubation filtrates of *C. coronatus* this technique was selected for qualitative and quantitative analysis of the trichothecenes mycotoxins (HT-2, T-2, deoxynivalenol, nivalenol) in obtained extracts. However, application of this technique for the determination of mycotoxins requires derivatization, which not only reduces the polarity of the compounds, but also enhances their volatility. In order to obtain thermally stable the effect of derivatizing reagent type, amount, reaction time, temperature of reaction, catalyst additive, or the type of solvent used. For derivatization of the hydroxyl groups present in the structure of the analyzed compounds derivatizing reagents used were as follows:

- Silylating: a mixture of 99% BSTFA (*N,O*-bis (trimethylsilyl) trifluoroacetamide) + 1% TMCS (trimethylchlorosilane), TMSI (*N*-trimethylsilylimidazole)
- Acylating: TFAA (trifluoroacetic anhydride with the sodium bicarbonate addition), AA (acetic acid anhydride with the anhydrous sodium acetate addition).

The reaction with a mixture of BSTFA + TMCS (99% + 1%) gave the stable derivatives of T-2 toxin and HT-2 when the reaction was carried out at 80 °C for 60 min. Whereas in case of using other reaction conditions (temperature 60 °C, 30 min.) no stable derivatives of T-2 toxin and HT-2 were obtained. Reaction of deoxynivalenol, nivalenol with TMSI also not afford to obtain stable derivatives. Then, the synthesis of acetyl derivatives were tested by reaction with TFAA or AA. The stable derivatives was obtained for T-2 and HT-2 toxins by reaction with acetic anhydride at 80 °C for 60 min. In the work of Mateo et al., derivatization of trichothecenes with different reagents was compared. The results shown that fluoroacylation of mycotoxins provides better results than trimethylsilylation for GC-ECD. Among acylation reagents, HFBA provided the highest toxin peak areas, although 15-AcDON was not derivatized. For derivatization of this toxin TFAA reagent with sodium bicarbonate is recommended giving also acceptable results for the other trichothecenes. In turn, our results indicate that stable derivatives of T-2 and HT-2 toxins was obtained with BSTFA:TMCS and acetic anhydride. However, procedure with BSTFA:TMCS does not require additional steps as in the case of the reaction with AA. In the case of NIV and DEO under the conditions tested did not receive derivatives. Therefore, the lack of a stable derivatives the use of GC-MS for the determination of trichothecenes proved impossible. Considering above for the determination of trichothecenes LC-MS has been optimized. The optimal parameters of the spectrometer allowing the isolation and detection of all

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analytes from the group of trichothecenes (T-2 toxin, HT-2 toxin, deoxynivalenol nivalenol) was chosen. In the case of the trichothecenes compounds (HT-2 toxin, and T-2 toxin, nivalenol and deoxynivalenol) in either positive ion and negative ion modes signals corresponding pseudo-molecular ions were obtained. The most intense signals at m/z 447.3 for HT-2 toxin and 489.4 for T-2 toxin were observed. Mass spectra for HT-2 toxin, and T-2 toxin, obtained by ionization in the negative ion mode signals are present at m/z, 423.2 and 465.4 respectively, corresponding to $[M-H]^-$ pseudo-molecular ions. In contrast, as a result of ionization in positive mode the presence of ions $[M + Na]^+$ for the HT-2 toxin, and T-2 toxin and ions $[M + H]^+$, $[M + NH_4]^+$ and $[M + K]^+$ for T-2 toxin were observed. In the mass spectra for nivalenol and deoxynivalenol, obtained by ionization in negative ion mode signals at *m/z* 351.1 and 335.1 were present, respectively. In contrast, as a result of ionization in the positive ion mode the presence of ions $[M + H]^+$ for nivalenol and $[M + Na]^+$ for deoxynivalenol was observed. Pseudo-molecular and fragmentation ions of the test compounds are shown in Table 1. The method was validated and selected validation parameters are shown in Table 2.

Optimization of extraction procedure

Several extraction solvent mixtures in different volumetric ratios have been employed for extraction of grain samples in published studies [14-20]. Due to the co-extractives typically present in the crude extracts obtained by a relatively polar solvent mixtures, high demands are placed on the efficiency of the step of cleaning, especially when relatively non-specific detection techniques were employed. On the basis of literature data four mixtures have been used for isolation of trichothecenes to check the best of them. To perform these studies lyophilized mycelium with and without analytes was assayed and all extracts were injected into the LC-MS system under the optimal conditions. The results of the comparative study are shown in Table 3. The mixture acetonitrile-water (85:15, v/v) seems to be the most suitable for extraction of type B trichothecenes from *C. coronatus* mycelium samples. The use of ethyl acetate-acetonitrile-water (77:19:4, v/v/v) provided the best extraction data for NIV, HT-2 and T-2 toxins but recovery value for DON was below 70 %. Both, methanol-water and acetonitrile-water in ratio 70:30 mixtures provided similar yields, but lower than with the other two solvents mixtures. Therefore to the extraction of the trichothecenes from *C. coronatus* mycelium and post-incubation filtrates the ACN:H_2O (85:15, v/v) mixture was applied.

Table 1: Summary of pseudo-molecular and the fragment ions of trichothecenes obtained by LC-MS

Mycotoxin	Pseudo-molecular ions	Ionization mode	Fragmentation energy [V]	Fragmentation ions
HT-2 Toxin	425,5 $[M+H]^+$	positive	1	240,2 301,3 340,3 408,5
	442.0 $[M+NH4]^+$	positive	1	175,3 233,2 425,4
T-2 Toxin	467,5 $[M+H]^+$	positive	1	169,2 177,1 257,3 283,3 363,3 407,4 451,5
	484.0 $[M+NH_4]^+$	positive	1	185,2 215,2 245,2 305,2 365,3 407,4 449,4 467,5
Nivalenol	313.0 $[M+H]^+$	positive	1	294.3 268.3 241.2
Deoxynivalenol	297.0 $[M+H]^+$	positive	1	95,6 166,2 279,3

Determination of trichothecenes in C. coronatus mycelium and post-incubation filtrates

As a result of LC-MS analysis of ACN:H_2O (85:15, v/v) extract of the *C. coronatus* mycelium grown on LB medium in optimal condition the signal corresponds to the HT-2 toxin were observed. Apart from the retention time, mass spectrum analysis was done to confirm the identification of this compound. The presence of pseudo-molecular ion at *m/z* 442 and fragment ions at *m/z*: 175.2, 233.2 and 425.4 clearly confirms the presence of HT-2 toxin in this extract. In the chromatogram obtained by LC-MS analysis of *C. coronatus* post-incubation filtrate extract signal corresponds to T-2 toxin was found. The initial identification was done by comparing of retention time obtained by LC-MS analysis of the extract and LC-MS analysis of T-2 toxin standard solution. In addition, identification has been confirmed by analysis of the mass spectra. The presence of the ion $[M + NH_4]^+$ at m/z 484.0, and the signals at m/z 185.2; 215.2; 245.1; 305.2; 365.1; 407.5; 467.5 allow a clear conclude that the T-2 toxin is present in this extract. In a similar way the presence of trichothecenes in the remaining mycelial and post-incubation filtrates extracts of *C. coronatus* was confirmed or ruled. In any of the tested extracts DON and NIV was not detected. The results are summarized in Table 4. *C. coronatus* produced higher amount of trichothecenes when is grown on LB medium. *C. coronatus* produced about 4-times more T-2 in temperature 20°C then in 30°C. The optimal temperature for production of both T-2 and HT-2 by *C. coronatus* was 20°C. However, the pH value is the same for grown of *C. coronatus* mycelium and trichothecenes production. Fungal homogenates exhibited strong effect on the cell viability of *G. mellonella* cells. There was decrease in the number of live cells after 24h of incubation (samples MM 20° pH 6, 7, 8, 9; MM 30° pH 9; LB 20° pH 7, 9; LB 30° pH 8), but Sf9 cells remained intact by these metabolites. However after 48 h of incubation almost all homogenates exhibited toxic activity on Sf9 cells (WST-1 test). Fungal homogenates exhibited strong effect on the cell viability of *G. mellonella* cells. There was decrease in the number of live cells after 24h of incubation (samples MM 20° pH 6, 7, 8, 9; MM 30° pH 9; LB 20° pH 7, 9; LB 30° pH 8), but Sf9 cells remained

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Table 2: Method validation parameters of the final determination of trichothecenes by using LC-MS.

Mycotoxin	Calibration curve	Coefficient of determination R^2	Accuracy [%]	Precision [%]	IQL [µg/ml]	IDL [µg/ml]
Nivalenol	y = 9E+07x - 5E+06	0,9931	98.4 – 102.8	4.72 – 5.21	0.06	0.02
Deoxynivalenol	y = 1E+09x - 1E+08	0,9971	95.8 – 99.5	5.42 – 7.13	0.06	0.02
T-2	y = 3E+09x + 7E+07	0,9991	89.6 – 107.3	3.85 – 4.56	0.03	0.01
HT-2	y = 7E+09x + 5E+08	0,9997	88.4 – 95.5	2.96 – 4.31	0.03	0.01

Table 3: Percentage of recovery for trichothecenes in spiked mycelium of *C. coronatus* by using LC-MS, RSD < 8%.

Spiking level [µg/g]	Extraction with ACN-H_2O (85:15, v/v)				Extraction with AcOEt:ACN: H_2O (77:19:4, v/v/v)			
	DEO	NIV	T-2	HT-2	DEO	NIV	T-2	HT-2
5	86.3	91.6	79.5	76.5	70.3	76.4	84.9	82.7
1	94.1	89.7	84.6	81.7	64.8	75.6	87.2	79.4
0.5	89.2	86.4	87.3	83.2	69.0	88.0	88.3	83.2
0.1	80.3	85.3	86.8	88.7	60.3	91.3	85.7	86.7
Spiking level [µg/g]	**Extraction with ACN-H_2O (70:30, v/v)**				**Extraction with MeOH: H_2O (70:30, v/v)**			
	DEO	NIV	T-2	HT-2	DEO	NIV	T-2	HT-2
5	55.7	63.9	68.8	58.4	63.2	57.8	48.3	45.6
1	53.8	60.3	60.4	59.7	59.4	51.4	50.5	48.3
0.5	50.9	65.6	57.5	53.6	45.2	52.8	49.6	40.6
0.1	47.8	59.4	55.7	57.9	42.3	49.6	45.9	46.2

Table 4: Trichothecenes concentration found in homogenates and post-incubation filtrates of *C. coronatus* cultivated at different conditions.

	Trichothecenes content [µg/g]				
The culture conditions	**Mass of *C. coronatus* mycelium [g]**	**DON**	**NIV**	**T-2**	**HT-2**
MM medium, optimal condition	7,11	nd	nd	1.6 ± 0.1	1.2 ± 0.3
LB medium, optimal condition	7,11	nd	nd	2.4 ± 0.1	4.2 ± 0.1
MM medium, pH 5, temperature 30 °C	0.49	nd	nd	nd	nd
MM medium, pH optimal, temperature 30 °C	0.87	nd	nd	<LOD	<LOD
MM medium, pH 5, temperature 20 °C	0.29	nd	nd	nd	nd
MM medium, pH optimal, temperature 20 °C	1.09	nd	nd	6.3 ± 0.2	4.5 ± 0.1
LB medium, pH 5, temperature 30 °C	0.51	nd	nd	nd	nd
LB medium, pH optimal, temperature 30 °C	0.99	nd	nd	0.9 ± 0.1	0.7 ± 0.1
LB medium, pH 5, temperature 20 °C	1.04	nd	nd	nd	nd
LB medium, pH optimal, temperature 20 °C	2.9	nd	nd	7.2 ± 0.2	5.9 ± 0.1
Post-incubation filtrate (MM)	100	nd	nd	3.7 ± 0.1	nd
Post-incubation filtrate (LB)	100	nd	nd	2.2 ± 0.2	nd

nd – not detected.

intact by these metabolites. However after 48 h of incubation almost all homogenates exhibited toxic activity on Sf9 cells (WST-1 test. The highest toxicity of *C. coronatus* filtrates were obtain when fungus was cultivation on MM medium in 30ºC and in pH 6, 7, 9. More than 50 % of insects were dead after 21 days of observation. It is confirmation of natural behavior of this fungus (tropical conditions: high temperature, high huminity) and that in stressful conditions, as MM medium is, this fungus secrets toxic metabolites. No effect of Post incubation filtrates on the cell viability of Sf9 cells and weak effect on the cell viability of *G. mellonella* cells were shown. There was slight decrease in the number of live cells after 24h of incubation (samples MM 20º pH 6, 7; MM 30º pH 5, 6), but Sf9 cells remained intact by these metabolites. However after 48 h of incubation filtrates with Sf9 cells indicated decrease in the number of live cells especially for MM samples (Table 5-9). The impact of environmental conditions on mycelial growth, toxicity and mycotoxin production of *Fusarium* species was studied. The optimal temperature for the production of mycotoxins by F. *sporotrichioides langsethiae* was lower than the growth of mycelium. This finding is disclosed in Hodgson (2000); who indicates that T-2 and HT-2 toxins in the form of secondary metabolites, should be produced under conditions which are not optimal for growth [21]. In vitro production of mycotoxins it is useful to predict the effect of temperature on the type and quantity of toxins produced by the infected plants

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Table 5: Effect of fungal filtrates, clean medium and IPS on *G. mellonella* larvae (tests *in vivo*).

Conditions of fungus cultivation	The dynamics of killing insects (% mortality)					Development stage of the dead insect
MM 20°C	**1 day**	**3 day**	**5 day**	**8 day**	**21 day**	**dead larvae / pupae**
pH 5	0	0	0	0	20%	0l 2p
pH 6	10	10	10	20	40%	2l 2p
pH 7	30	40	40	40	**70%**	4l 3p
pH 8	0	20	20	20	**50%**	2l 3p
pH 9	0	10	10	10	20%	1l 1p
MM 30°C	**1 day**	**3 day**	**5 day**	**8 day**	**21 day**	**dead larvae / pupae**
pH 5	0	0	0	0	20%	0l 2p
pH 6	20	40	50	50	**70%**	5l 2p
pH 7	10	20	30	30	**50%**	3l 2p
pH 8	10	20	20	30	40%	3l 1p
pH 9	20	50	50	50	**80%**	5l 3p
LB 20°C	**1 day**	**3 day**	**5 day**	**8 day**	**21 day**	**dead larvae / pupae**
pH 5	0	0	0	0	0	0l 0p
pH 6	30	30	30	30	30%	3l 0p
pH 7	0	0	0	0	30%	0l 3p
pH 8	10	20	20	20	**50%**	2l 3p
pH 9	10	10	10	10	20%	1l 1p

Table 6: Effect of fungal homogenates on *G. mellonella* larvae (tests *in vivo*).

Conditions of fungus cultivation	The dynamics of killing insects (% mortality)					Development stage of the dead insect
MM 20°C	**1 day**	**3 day**	**5 day**	**8 day**	**21 day**	**dead larvae / pupae**
pH 5	0	0	0	20	70%	2l 5p
pH 6	10	30	30	40	90%	5l 4p
pH 7	10	40	40	40	90%	5l 4p
pH 8	20	20	20	20	80%	2l 6p
pH 9	60	70	70	70	70%	7l 0p
MM 30°C	**1 day**	**3 day**	**5 day**	**8 day**	**21 day**	**dead larvae / pupae**
pH 5	0	0	0	10	40%	1l 3p
pH 6	10	10	10	10	60%	3l 3p
pH 7	10	20	20	30	40%	4l 0p
pH 8	10	30	30	40	70%	4l 3p
pH 9	20	30	30	30	70%	4l 3p
LB 20°C	**1 day**	**3 day**	**5 day**	**8 day**	**21 day**	**dead larvae / pupae**
pH 5	20	20	20	20	60%	3l 3p
pH 6	0	10	30	40	60%	4l 2p
pH 7	30	30	30	50	70%	5l 2p
pH 8	50	50	50	50	70%	5l 2p
pH 9	50	60	60	70	80%	7l 1p
LB 30°C	**1 day**	**3 day**	**5 day**	**8 day**	**21 day**	**dead larvae / pupae**
pH 5	10	30	30	30	80%	5l 3p
pH 6	10	20	20	50	90%	5l 4p
pH 7	10	20	30	50	100%	8l 2p
pH 8	20	20	20	40	60%	4l 2p
pH 9	20	40	40	50	70%	5l 2p
IPS	10	10	20	30	30%	3l 0l
KK	10	20	20	20%		21

Table 7: Effect of fungal filtrates on the proliferation of Sf9 cell (in vitro test - WST-1).

The culture conditions of the fungus	% dead cells		The culture conditions of the fungus	% dead cells	
MM 30°C	**25 µl**	**50 µl**	**LB 30°C**	**25 µl**	**50 µl**
pH 5	33	51	pH 5	8,3	57
pH 6	37	24	pH 6	0	0
pH 7	18	0	pH 7	19	32
pH 8	0	0	pH 8	10	13
pH 9	0	0	pH 9	0	0
MM 20°C	**25 µl**	**50 µl**	**LB 20°C**	**25 µl**	**50 µl**
pH 5	15	22	pH 5	41	51
pH 6	12	19	pH 6	23	30
pH 7	60	10	pH 7	0	0
pH 8	0	0	pH 8	0	0
pH 9	0	10	pH 9	15	22

Table 8: Effect of fungal filtrates on *G. mellonella* and Sf9 cell viability (*in vitro* test - calcein).

The culture conditions of the fungus	% dead cells		The culture conditions of the fungus	% dead cells	
Homogenat MM 20°C	**100 µg/ml**	**250 µg/ml**	**Homogenat LB 20°C**	**100 µg/ml**	**250 µg/ml**
pH 5	30	0	pH 5	0	0
pH 6	5	8	pH 6	0	0
pH 7	13	26	pH 7	20	22
pH 8	27	25	pH 8	0	6
pH 9	10	17	pH 9	18	10
Homogenat MM 30°C	**100 µg/ml**	**250 µg/ml**	**Homogenat LB 30°C**	**100 µg/ml**	**250 µg/ml**
pH 5	6	1	pH 5	6	0
pH 6	5	3	pH 6	0	0
pH 7	10	0	pH 7	0	0
pH 8	0	0	pH 8	6	18
pH 9	15	27	pH 9	0	10

Table 9: Effect of fungal homogenates on Sf9 cell proliferation (*in vitro* test - WST-1).

The culture conditions The culture conditions of the fungus % dead cells of the fungus % dead cells					
MM 20°C	**100 µg/ml**	**250 µg/ml**	**MM 30°C**	**100 µg/ml**	**250 µg/ml**
pH 5	18	85	pH 5	7	20
pH 6	20	36	pH 6	0	10
pH 7	0	87	pH 7	13	13
pH 8	0	0	pH 8	37	15
pH 9	4	57	pH 9	38	77
LB 20°C	**100 µg/ml**	**250 µg/ml**	**LB 30°C**	**100 µg/ml**	**250 µg/ml**
pH 5	17	24	pH 5	0	30
pH 6	40	60	pH 6	22	35
pH 7	15	24	pH 7	32	24
pH 8	7,4	0	pH 8	7	0
pH 9	0	0	pH 9	10	4

and contamination of the products derived from them [22]. Production of T-2 and HT-2 by these two species can be at relatively low temperatures, i.e., the highest toxin production at 10 and 15 °C. While the low temperature have been previously proposed to be optimal for the production of trichothecene by *F.*

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sporotrichioides and *F. langsethiae*, wider range of temperatures was tested in the study than in previous studies. In the work of Marasas et al. (1987) and Kokkonen et al. (2010), 15 °C was more favorable than 25 °C to trichothecene-type production [22,23]. Park et al. (1993), reported that 15 °C is more favorable than 25 °C to T-2 production as well as on the general type of trichothecenes production by *F. sporotrichioides* [24]. In Mateo et al. (2002), 20 °C (instead of 26 or 33 °C) was the most appropriate temperature T-2, the preparation and production of trichothecene in general [25]. The authors found that the interaction with the substrate, temperature and humidity were too complicated to determine their precise impact on the production of each type of trichothecene. *F. sporotrichioides* was more toxic than *F. langsethiae* and both species produced more HT-2 than T-2. However, the ratio of T-2 and HT-2 changed with temperature. Mylona and Mangan (2011) found that the sum of the two toxins were higher at 25 °C than at 15, 20 or 30 °C under tested conditions (0.89, 0.945 and 0.97), which indicates that 25 ° C may be optimal temperature to production of T-2 and HT-2 toxin by *F. langsethiae* [26]. The results obtained by Nazari were differ from those described by Doohan et al. (2003), who reported that *F. sporotrichioides* most toxins produced at intermediate temperatures (20 and 26 °C; 20, 26 and 33 °C were tested) in very humid conditions (0.990 aw; aw values of 0.990, 0.995 and 0.999 were tested) [22, 28]. Nazari et al., (2014) found that the optimal temperature range for growth was 20-25 °C for *F. langsethiae* and 25-30 °C for *F. sporotrichioides*, and the optimum for production of both T-2 and HT-2 toxins was 15 °C for *F. langsethiae* and 10-15 °C for *F. sporotrichioides* [28]. Floret infection occurred from 10 to 40 °C for *F. sporotrichioides* (69.8% average incidence of infected florets) and from 10 to 35 °C for *F. langsethiae* (17.6% of infected florets). The optimal temperature for spike colonisation was 25 °C for *F. langsethiae* and 30 °C for *F. sporotrichioides*, and the optimal temperature range for mycotoxin production was 15-35 °C for *F. langsethiae* and 20-25 °C for *F. sporotrichioides*. Medina and Magan (2011) also observed changes in this indicator under stress conditions *in vitro* [29,30]. Kokkonen et al. (2010) reported that *F. langsethiae* produced more T-2 than the HT-2, while Langseth and Rundberget (1999) found higher than the HT-2, T-2 in oats and other grains stored in a conditions probably unfavorable for the growth of *Fusarium* species [22,31].

Conclusion

C. coronatus is little explored cosmopolitan soil fungus showing high insecticidal potential. Information on the mycotoxin production of *C. coronatus* is essential for a better understanding of the mechanisms leading to the insecticidal activity. In this work identification of toxic metabolites from trichothecene group produced by the parasitic fungus *C. coronatus* was done. The results of our study allow conclude that the T-2 and HT-2 toxins were present in *C. coronatus* extracts. The effect of culturing conditions on production of trichothecenes were determined. The optimal temperature for production of both T-2 and HT-2 by *C. coronatus* was 20°C. It was also shown that *C. coronatus* produced higher amount of trichothecenes when is grown on LB medium However, the pH value is the same for grown of *C. coronatus* mycelium and trichothecenes production. In any of the tested extracts DON and NIV was not detected. Toxicity of *C. coronatus* homogenates and post-incubation filtrates to *G. mellonela* larvae and Sf9 cells was also determined. The obtained results provide a good framework for further research on the insecticidal potential of *C. coronatus*. It is necessary to conduct further research compounds produced by *C. Coronatus* grown under optimal conditions and stress and to determine the impact of growing conditions for the synthesis of mycotoxins by filamentous fungi and their virulence potential.

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