UNIVERSITY OF DEBRECEN
Centre of Agricultural Sciences
Faculty of Agriculture
Department of Plant Protection

**INTERDISCIPLINARY AGRICULTURAL AND NATURE
SCIENCES Ph.D. PROGRAMME**

Head of Ph.D. programme:
Prof. Dr. Nagy János
Doctor of the Hungarian Academy of Sciences (DSc)

Supervisor:
Dr. habil. György Kövics
Associate Professor of Plant pathology

„THESIS OF THE Ph.D. DISSERTATION"

SPECIES AND STRAIN SPECIFIC GLIOTOXIN

PRODUCTION OF *TRICHODERMA*

Written by:

Péter Harcz
PhD candidate

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1. Main objectives of the research work

In this study, ninety-six different strains of eleven *Trichoderma* species located at the MUCL collection, were screened for gaining insight into their isolation, morphological and molecular biological characterisation, gliotoxin producing potential. Gliotoxin were studied as a metabolite playing role in biological control, and like mycotoxin as well.

In this work, numerous *Trichoderma* strain were fermentated in laboratory scale systems, and the produced gliotoxin were detected by HPLC system. 17 screened strains were originated from the collection of the UDCASFA Department of Plant Protection. and from other collections as well.

Morphological and molecular biological identification and comparison (RAPD PCR and ITSrDNA sequencing) were done to find the taxonomical position of gliotoxin producing fungi within the genus *Trichoderma*.

In vitro experiment were established for gaining insight into the role of gliotoxin in antagonism against the pathogen *Rhizoctonia solani*.

2. Premises of the research work

Fungi of the genus *Trichoderma* have been known for more than 200 years. *Trichoderma* fungi are characterized by rapid growing colonies, cosmopolitan and frequently dominant in soils, on decaying wood and on vegetable matter, due to the diverse metabolic capability of the species and its aggressively competitive nature. Indeed, mycoparasitic *Trichoderma* strains are able to recognize the host hyphae, to coil around them, to develop haustoria for penetration of the host cell using cell wall degrading enzymes like chitinases, glucanases and proteases, and to utilize the contents of the host hyphae as nutrient source. The mycotoxin and antibiotic gliotoxin is one of the well-known member of the epipolythiodioxopiperazine (ETP) class of fungal metabolites. The production of this metabolite is establised in the genus *Trichoderma* since 1930's. In the last 80 years the gliotoxin producing *Trichoderma* thought to be different species, due to the not well established *Trichoderma* taxonomy and due to the misidentifications. In papers and publications several name of the gliotoxin producing fungi can be found: *Trichoderma*

lignorum, *Trichoderma viride*, *Gliocladium virens*, *Gliocladium fimbriatum*, *Trichoderma virens*. The name 'gliotoxin' can be also originated from a mistake, since the producing fungi was thought to be *Gliocladium* sp. with green conidia.

In the literature just 1 (2) strains (Gl 20, Gl 21) can be found in gliotoxin producing experiments. There are still dissents in the role of gliotoxin in antagonism and antibiosis, where as the strong antimicrobial attribute of this toxin is well known. In some studies, gliotoxin production by *Trichoderma* is considered as an important trait to achieve biological control.

3. Materials and methods

Trichoderma strains

Ninety-six *Trichoderma* strains were provided by the "Mycothèque de l'Université catholique de Louvain (MUCL, Belgium) which is a member of the Belgian Coordinated Collection of Microorganisms (BCCM[TM]) consortium founded by the Belgian Science Policy. Among these strains, seventeen were previously originated from the collection of the Centre for Agricultural Sciences (Department of Crop Protection, Faculty of Agronomy, University of Debrecen, Hungary). These strains had different geographic and substrate origins and belong to eleven *Trichoderma* species: *T. harzianum (17), T. viride (17), T. longibrachiatum (12), T. virens (11), T. koningii (10), T. polysporum (8), T. hamatum (6), T. pseudokoningii (6), T. atroviride (3), T. aureoviride (4), T. flavofuscum (1)*. A preliminary study was done to determine the growth rate and the gliotoxin production in different synthetic and complex liquid culture media like 2% malt extract broth and the synthetical medium optimized for gliotoxin production. All these media were initially at 121°C for 15 min and stored at 4°C until use.

Trichoderma strains from one-week old cultures were harvested in sterile water and the initial number of conidia and chlamydospores of the suspension was counted in a haemacytometer at 400× magnifications with a light microscope. The media (50ml) used for the fermentation in 100ml-Erlenmeyer flasks was then inoculated to reach the inoculum size of 10^3 conidia ml^{-1} of medium.

The flasks were shaken at a speed of 95 min^{-1} in a thermostatically controlled room at 25°C for corresponding

duration of incubation (62 and 88 h in the case of the screening of the 96 strains; 16, 24, 40, 48, 64, 72, 88 and 96 h in the case of the set up of the kinetics). After fermentation, the supernatant (1 ml) was taken from the liquid phase of the culture with a sterile pipette and freeze-stored in 1.5 ml-Eppendorf tubes at $-18°C$ until gliotoxin analyses. These fermentation experiments were made in four replicates (two replicates at two different periods) for each strain.

The chromatographic system consisted of a Waters 510 pump equipped with an automatic WISP model 712 injector, a Diode array detector set at 270 nm. The signals of the detector were processed and visualized by computer using the ChromQuest 3.0 software. The analytical column was a Chromsep SS Microsphere C_{18} column with a CP 28141 precolumn. The column was maintained at 35°C by a temperature controller model SparkHolland SpH99 during HPLC analyses. The mobile phase was made of a mixture of HPLC grade acetonitrile:water (75:25), filtered through a 0,22 μm filter membrane and degassed. The flow-rate was fixed at 1.0 ml min^{-1} and the injection volume was 25 μl.

2% malt extract broth were used for mycelial biomass production. For DNA extraction, purchased DNA extraction KIT was applied which method is based on standard methods. Amplifications were carried out with random primers of 10 nucleotides in the case of RAPD.

For the ITSrDNA sequence were amplified by primers LR1 (5'-GGTTGGTTTCTTTTCCT-3') and SR6R (5'-AAGTAGAAGTCGTAACAAGG-3'). The sequencing were carried out at MWG Biotech GmbH, Germany. The alignmet and the phylogenetical analyses of the sequences were made by using ClustalX, MEGA2 and GeneDoc softwares, and the tree was made by TreeView software.

4. Main statements of the dissertation

Results of RAPD PCR analyses

For each tested strain, amplification with the four random primes allows the generation of reproducible amplified patterns. The patterns of all these results are standardized and analyzed by the phenetic analysis.

The total number of reproducible polymorphic bands generated by the amplification with the four random primers allowed analysing the similarity among the *T. virens* strains. Checked with the Pearson correlation test, the phenogram (Figure 1.) did not show correlation between the gliotoxin production and the results of the RAPD analyses, and between the substrate origin and the RAPD results. The geographical origin of the strains did not show any correlation with the gliotoxin production neither with the genetic diversity.
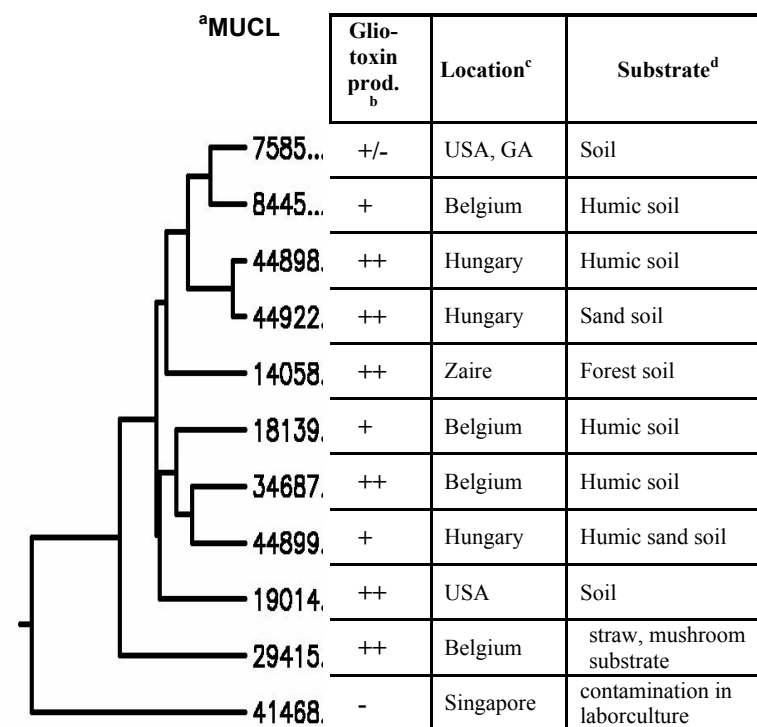

[a]MUCL	Glio-toxin prod.[b]	Location[c]	Substrate[d]
7585...	+/-	USA, GA	Soil
8445...	+	Belgium	Humic soil
44898.	++	Hungary	Humic soil
44922.	++	Hungary	Sand soil
14058.	++	Zaire	Forest soil
18139.	+	Belgium	Humic soil
34687.	++	Belgium	Humic soil
44899.	+	Hungary	Humic sand soil
19014.	++	USA	Soil
29415.	++	Belgium	straw, mushroom substrate
41468.	-	Singapore	contamination in laborculture

Figure 1.: Results of phenetic analyzes (dendrogram) of the RAPD results with the 11 *T. virens* strains
[a]MUCL: Mycothèque de l'Université catholique de Louvain (member of the „Belgian Coordinated Collections of Microorganisms BCCM[TM] consortium" Louvain-la-Neuve, Belgium)
[b]Gliotoxin production: +/-: traces; +: low-producing strains; ++: high producing strains; paying respect to the peak production of the high-producing strains at 64h, as point of reference
[c]location: the geograpical origin of the strains
[d]substrate: the original substrate of the strain

Results of the ITSrDNA analyses

Analyses of ITSrDNA sequences of the tested 3 representative gliotoxin producing strains did not show differences. At the same time, the other non gliotoxin-producer species can be well separated by using ITSrDNA sequences (Figure 2.).
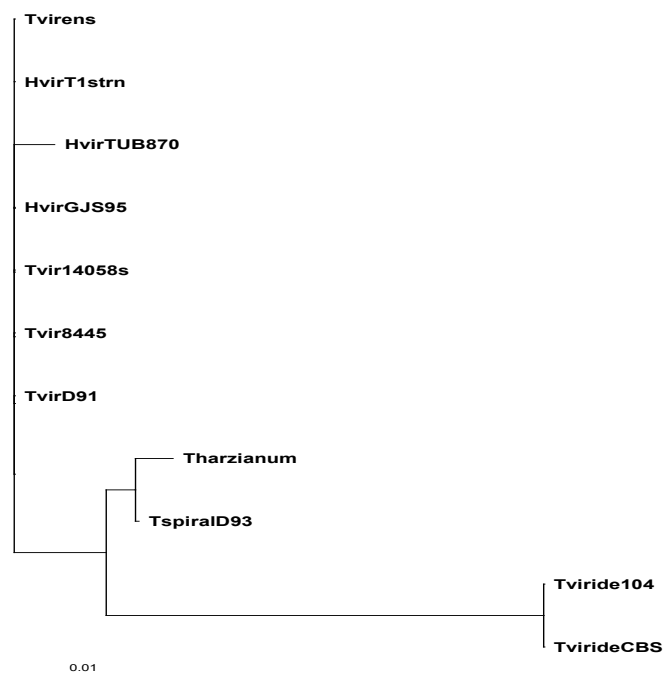

Figure 2. Phylogenetic analysis of ITS1 and ITS2 rDNA (phylogram). Tvir8445, TvirD91 and Tvir 14058 are representatives of 3 gliotoxin producing *T. virens* strains. Tvirens, HvirT1strn, HvirTUB870, HvirGJS95: representaive T. virens ITS1-2 sequences from the NCBI sequence collection. Tharzianum, Tspiral, Tviride104, TvirideCBS: ITS1-2 sequences of different species.

Results of gliotoxin producing experiments

Preliminary survey performed with fifteen strains to determine the growth rate and the gliotoxin production by *Trichoderma* strains in two media had shown that significantly higher ($P < 0,001$) intensive growth and gliotoxin production were observed in malt broth than in gliotoxin medium (Table 1.). All the tested strains (gliotoxin and non-gliotoxin producers) have shown intensive or moderate growth on malt broth (2 %) during the fermentation periods. Malt extract medium was then used for further experiments.

Comparing the gliotoxin production in malt medium within 62 and 86 h, the Wilcoxon Signed Ranks test did not revealed any statistically significant change in gliotoxin production ($0,068 \leq p \leq 1,000$). Nevertheless, it was observed slightly decreases in gliotoxin production upon additional incubation for 24 h (Table 1), showing thus the temporal fluctuation of this metabolite production. For some strains, it was observed that increasing the duration of incubation up to 86 h, led to decreasing of glitoxin production level. However, the reverse trend was also observed for other strains, demonstrating a strain-specific variability for gliotoxin production. We therefore performed

a screening study to find out the suitable strains able to produce gliotoxin and a kinetic study to check the appropriate incubation time for significant gliotoxin production.

Screening of Trichoderma *strains for gliotoxin production*

The ninety-six *Trichoderma* strains were tested for their ability to produce gliotoxin upon incubation during 86 h. Only ten strains, all belonging to the *T. virens* species were repetitively found to produce gliotoxin at levels up to 49,6 μgml^{-1} upon incubation at 25°C during trials. Among *T. virens* strains, MUCL 7585 produced only trace level of gliotoxin while MUCL 41468 did not produce detectable amount of gliotoxin, showing once again the variability within this species in the capability of production of this secondary metabolite.

Using nine gliotoxin-producing strains, experiments were set up for studying the biomass production, pH changes in the medium in relation to the kinetics of gliotoxin production as depicted in figures 2 and 3, for two representative strains.

T. virens strains had shown intensive growth with the mycelial dry weights were ranged between 208-241 mg upon incubation during 96 h. No change in the pH values of the control malt broth (4.8-5.0) was observed along the incubation period. Upon incubation, this value decreased up to $4,0 \pm 0,5$ until 40 - 48 h and then increased to reach $7,0 \pm 0,5$ by the end of the fermentation period. Interestingly, the changes in pH values are in accordance with the growth and the differentiation patterns of the fungi.

Gliotoxin production changed significantly for the tested strains (48,5 μgml^{-1} in the case of MUCL 34687 against 8 μg ml^{-1} in the case of MUCL 8445) and similarly the maximum level of the metabolite production occurred at various periods upon the incubation during the same period.

Table 1.: Effect of the culture media on the mycelial dry weight of *Trichoderma* and level of gliotoxin production upon incubation at 25°C during 62 and 86 h.

	Broths					
	Malt broth[b]			Gliotoxin producing broth[b]		
MUCL strains	Mycelial dry weight	Gliotoxin production[c]		M. dry weight.	Gliotoxin production[c]	
Nr.[a]	(mg)	62 h	86 h	(mg)	62 h	86 h
8445	128 ± 10	18,1±10,1	18,6 ± 6,8	65 ± 7	4,2 ± 1,0	6,2 ± 0,9
14058	145 ± 6	52,5 ± 5,7	49,6 ± 6,9	32 ± 6	14,6 ± 3,1	22,9 ± 2,4
18139	126 ± 10	17,1 ± 5,0	21,7 ± 5,9	35 ± 5	2,1 ± 0,4	2,4 ± 0,4
19014	104 ± 17	26,5 ± 3,9	33,3 ± 6,2	30 ± 6	1,0 ± 0,01	1,0 ± 0,1
29415	149 ± 8	32,7 ± 4,8	32,7 ± 5,4	50 ± 5	6,7 ± 1,9	10,8 ± 2,0
34687	145 ± 8	47,8 ± 4,4	26,3 ± 4,6	45 ± 10	10,0 ± 1,0	10,3 ± 1,1
41468	168± 9	ND	ND	51 ± 5	ND	ND
44898	148 ± 9	48,5 ± 4,4	44,2 ± 4,3	51 ± 6	4,0 ± 0,3	5,2 ± 0,3
44899	151 ± 10	16,5 ± 2,9	5,0 ± 1,1	27 ± 7	1,1 ± 0,01	1,97 ± 0,2
44922	167 ± 7	31,0 ± 1,8	35,0 ± 4,9	-	-	-
8965	141 ± 4	ND	ND	24 ± 6	ND	ND
11316	117 ± 8	ND	ND	35 ± 5	ND	ND
29736	169 ± 8	ND	ND	46 ± 6	ND	ND
44905	137 ± 4	ND	ND	59 ± 7	ND	ND
44906	185 ± 8	ND	ND	55 ± 5	ND	ND

[a] MUCL: Mycothèque de l'Université catholique de Louvain (member of the Belgian Coordinated Collections of Microorganisms BCCM[TM] consortium, Louvain-la-Neuve, Belgium

[b] : mycelial dry weight and gliotoxin production after 62 and 86 h incubation. in malt broth, significantly higher (p < 0.001) than gliotoxin producing sythetical media

[c]: Gliotoxin production, µg ml^{-1} broth

ND: not detectable (gliotoxin concentration in broth < 0,1 µg ml^{-1})

The growth phase appropriate for gliotoxin production was determined by comparing the relative timing of gliotoxin production and the fungal cell growth in liquid culture. The specific gliotoxin production related to the mycelial dry weight gives evaluable and comparable result of the gliotoxin production during the laboratory scale fermentation period. Using the gliotoxin production patterns, two groups of strains were formed. The group of strains MUCL 14058, 19014, 29415, 34687, 44898, and 44922 produced greater amount of gliotoxin than the other group of strains (MUCL 8445, 18139 and 44899) (Figure 3.). Timing of gliotoxin production presented also a significant difference. Indeed, in the case of the great producers, the maximum level of the synthesis occurred within 48-64 h upon incubation whilst the low producers had just slightly increasing production during the whole incubation period, close to the initial gliotoxin concentration(Figures 4-5.).
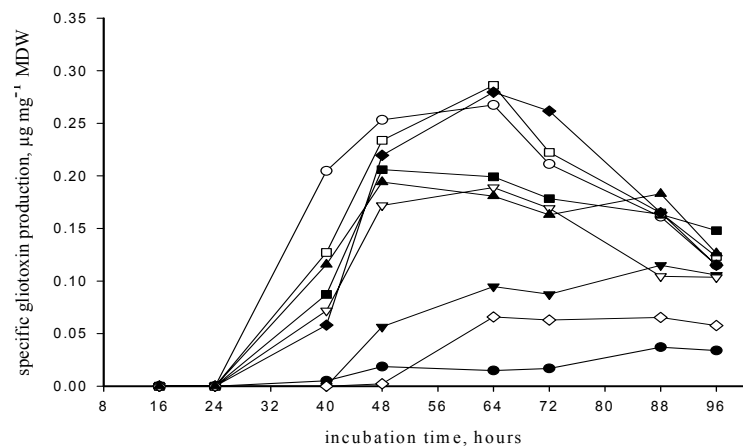
Figure 3: Kinetics of nine gliotoxin producing *T. virens* strain's gliotoxin production. Respectively: MUCL 8445 (●), 14058 (○), 18139 (▼), 19014 (▽), 29415 (■), 34687 (□), 44898 (◆), 44899 (◇), 44922 (▲) (in 2% malt broth). MDW: mycelial dry weight
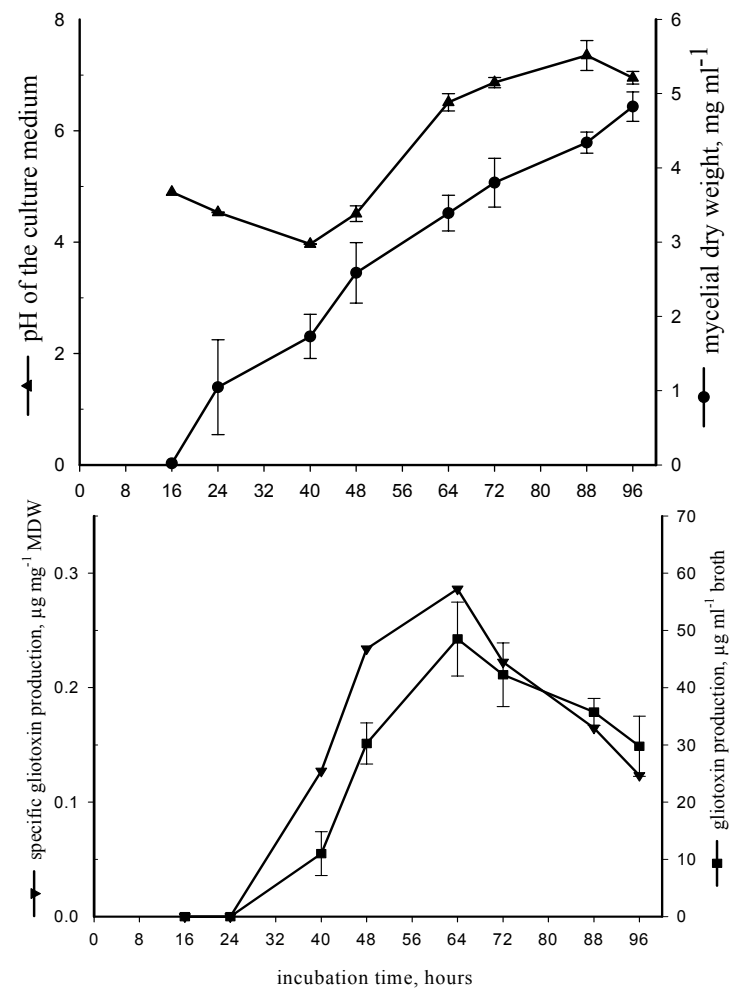

Figure 4: Dry weight production (●), pH of the culture medium (▲), specific (▼) and cumulative (■) gliotoxin production, and the kinetics of gliotoxin production in the case of MUCL 34687 Trichoderma virens strain (in 2% malt broth). MDW: mycelial dry weight
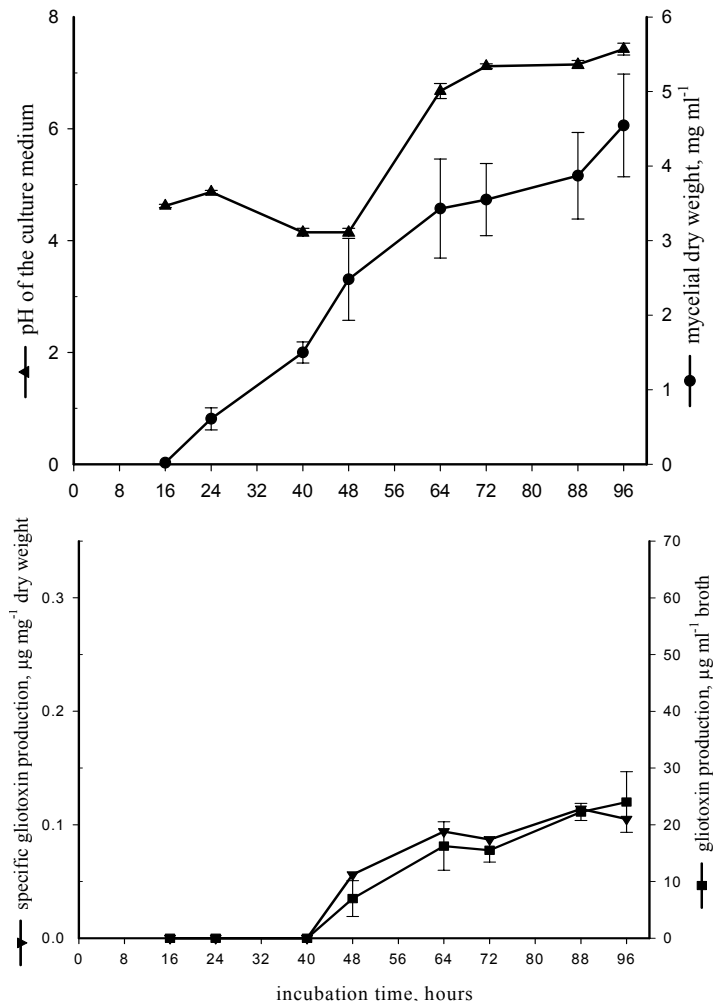
Figure 5: Dry weight production (●), pH of the culture medium (▲), specific (▼) and cumulative (■) gliotoxin production, and the kinetics of gliotoxin production in the case of MUCL 18139 *Trichoderma virens* strain (in 2% malt broth). MDW: mycelial dry weight

Results of the confrontation antagonism test

The radial growth of the pathogen's colony were measured every four hours. After the stop of pathogen's radial growth toward the antagonist's colony, the zone of inhibition were recorded. The mean of the inhibition zones were compared uniformly with the gliotoxin production at the 64th hour of fermentation. Statements of statistical significance were based on $p < 0,05$. Pearson correlation test was used to find correlation between the strain specific gliotoxin production level and the size of the inhibition zone.

No correlation were found with the SPSS 10.0.5 program package. This result allude to the lack of links between the gliotoxin production and the biological control activity.

Differences in the appereance of inhibition and in the sizes of the inhibition zones can be resulted by the inhibition effect of other (not measured) antimicrobial metabolites and of the different growth intensity of the screened strains.

5. New and patent results of the dissertation

- Ninety-six *Trichoderma* strains were tested for their ability to produce gliotoxin. Only ten strains (10,4%), all belonging to the *T. virens* species were repetitively

found to produce this metabolite. This work proofed and reconfirmed that fact just *T. virens* strains could produce this metabolite in detectable amount. We could not find strains are representing other species were producing gliotoxin.

- Furthermore, differences in timing and the kinetic patterns of gliotoxin production were observed among the gliotoxin producing strains.

- High-producer of gliotoxin showed the optimum production ability within 48 – 64 h against a steady state of the metabolite production by low-producer strains. Gliotoxin synthesis increased with fungal biomass and thereafter, it declined in the case of the high-productive strains.

- No significant correlation between the amount of gliotoxin production and the results of RAPD analyses was found.

- It was not feasible to separate gliotoxin producing *T. virens* strains from eachother by using ITSrDNA sequence analyses. Non gliotoxin producer *Trichoderma* strains were separated from other species by ITSrDNA sequence analyses. *T. viride*, which fungus were earlier

thought to be a gliotoxin producer was also separated by ITS sequences.

- No significant correlation between the giotoxin production and the inhibitory effect against *Rhizoctonia solani* was found.

6. Availability of the results in practice

This work confered about biological control organism *Trichoderma virens*. The antibiotic production of *Trichoderma virens*, – which is a frequent soil fungi and can be found in commercial biological control products as well – were studied in this dissertation.

This well known antibiotic gliotoxin and the production of the metabolite were studied from different aspects. Results of the experiments revealed that gliotoxin production can be diverse within isolates of *T. virens* used in biological control.

Results of *in vitro* antagonism test showed that the gliotoxin did not play the main role in antibiosis. In soil environment, the role of gliotoxin can also be doubtful, due to the transient production and the instability of the molecule.

Commercial products containing *T. virens* can be harmless for the manufacturer, or for the farmer, due to the speciality

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of the production and the chemical instability of gliotoxin, the dry product does not contain this metabolite.

This experiment pointed to the morphological variance and to the differences in the gliotoxin producing ability. These differences raise an issue about the uniformity of *Trichoderma virens* species. The ITS sequence was not specific to separate strains within *T. virens* species. By using other more specified sequences of *T. virens* can make new perspectives for the taxonomy of this species.

7. Publications

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