NKFI K-116475: *Trichoderma* green mould disease of cultivated mushrooms from the genomics perspective – closing report

The *Trichoderma* strains involved in the project were from 3 *Trichoderma* species causing green mould diseases of cultivated mushrooms: *Trichoderma pleuroti* TPhu1 and *Trichoderma pleuroticola* A37, two green mould isolates derived from *Pleurotus ostreatus* cultivation substrate (wheat straw) at a mushroom farm in Kecskemét, Hungary, and *Trichoderma aggressivum* f. *europaeum* CBS 433.95 isolated in 1991 from *Agaricus* compost in Northern Ireland. Genomic DNA was isolated from the 3 *Trichoderma* green mould species with the Qiagen DNeasy® Plant Mini Kit. The species-level identity of the green mould isolates was confirmed by the DNA Barcoding of the ITS (internal transcribed spacer of the ribosomal RNA gene cluster) and *tef1* (translation elongation factor 1α) markers with the tools *TrichO*key, *Tricho*BLAST, and NCBI BLAST. DNA libraries were prepared with NEBNext® Fast DNA Library Prep Set for Ion Torrent. The optimization of the Ion Torrent NGS platform for fungal genome sequencing was performed with *T. pleuroti* TPhu1. For the preparation of genomic DNA library, size selection was performed with E-Gel Electrophoresis System on 2% E-Gel® SizeSelect™ Agarose Gel. Size and quality of gDNA libraries were verified by Agilent DNA High Sensitivity Kit on Agilent 2100 Bioanalyzer. Genomic DNA library was firstly prepared using 200 bp insert size and sequenced on Ion 316 chip v2, resulting in 845 Mbp in 4,154,077 reads. These data were insufficient for *de novo* genome assembly, therefore we prepared a library with the 400 bp insert size protocol and used 316 chip v2 for sequencing, which resulted in 674 Mb in 1,787,798 reads. The two datasets provided about 41-fold coverage. Genome sequencing was performed with the optimized protocol also for *T. aggressivum* and *T. pleuroticola*. To refine the IonTorrent genome sequence data, Illumina Paired End (2×150 bp) sequencing was performed on the 3 mushroom green mould pathogens. The Illumina data were completed with MinIon sequencing to scaffold larger regions from the short, unique sequences. The resulting reads were demultiplexed (PoreChop) and the adapter regions removed (Trim Glamor), which was followed by quality-based filtering with unique scripts (length, quality, repetitive regions), so that only the good quality reads are used during the genome assembly. The usual check was performed with FastQC. *De novo* genome assemblies were carried out with SPAdes Genome Assembler 3.6.0 with the k-mer sizes of 33, 55, 77, 99 and 127. For the *in silico* identification of the genes, contigs were submitted to the Augustus 3.2.2 gene prediction software trained with the *T. virens* genome sequence. Results are shown in Table 1.

Table 1. Main characteristics of the 3 green mould genomes based on multiplatform data

Species	**Number of contigs**	**Genome length (Mbp)**	**G+C (%)**	**No. of CDS**
T. pleuroti	753	38.1	49.2	11322
T. pleuroticola	292	38.7	49.4	11054
T. aggressivum	117	38.6	48.9	11337

During functional annotation, the intracellular localization of the proteins was characterized with several tools, SignalP 5.0 was used to predict signal sequences, TMHMM 2.0 to identify the helix structure regions of proteins spanning membranes, DeepLoc 1.0 to predict the localization of the proteins in cell organelles with a neuronal network, while GPI-

anchored proteins were detected with NetGPI 1.1. Annotations were performed with BLASTP based on homology with proteins in several structured databases, including TCdb, MEROPS, Cythocrome P450, PHI, CAZymes. InterPro analysis was performed including the tools TIGRFAM, SFLD, ProDom, Hamap, SMART, CDD, ProSiteProfiles, ProSitePatterns, SUPERFAMILY, PRINTS, PANTHER, Gene3D, PIRSF, Pfam, Coils and MobiDBLite. KEGG annotation was based on KofamKOALA (HMM profiles) and BlastKOALA (homology search), predicting GO, KO, EC numbers and metabolic pathways. NapDos was used to predict secondary metabolite genes, while Mitos2 for the prediction of mitochondrial genes.

Predicted protein sets from the 3 green mold species were submitted to Ortho Venn with 3 non green mold species (*T. harzianum*, *T. reesei* and *T. virens*). Ortholog proteins grouped only to green mold species were submitted to BLAST2GO v 5.2.0 to search homology (BLASTp against non-redundant protein database) and functional annotations were determined by InterPro terms. A total of 11790 orthologous clusters were formed based on the protein sequences from the 6 species and 7324 gene clusters are shared by all 6 species. 90 proteins specific to green mold species were identified: 15 with ion-binding, 9 with binding, 9 with transferase, 8 with hydrolase, 8 with nucleotide-binding, 7 with nucleoside-binding, 7 with nucleic acid-binding, 5 with protein-binding activity and 22 with molecular function.

For phylogenetic analysis, 100 proteins from the 3 green mold species and 12 reference *Trichoderma* genomes were selected. Protein sequences were concatenatad and aligned by MAFFT v7. Poorly aligned and divergent regions were eliminated by Gblocks and the best substitution model was selected by SMS in PhyML. Bayesian analysis was performed by MrBayes v3.2.6 with Dayhoff I+G+F amino acid substitution model, which confirmed the position of the newly sequenced *Trichoderma* strains within clade Harzianum of the genus.

Identification of secondary metabolite producing enzymes was performed by SMIPS and polyketide synthases (PKS) as well as nonribosomal peptide synthases (NRPS) were predicted, which resulted in 14, 15 and 15 NRPS, 21, 26 and 26 PKS, as well as 6, 8 and 8 NRPS-PKS hybrid enzymes in *T. pleuroti*, *T. aggressivum* and *T. pleuroticola*, respectively. A 14-module and an 18-module NRPS-PKS hybrid were also identified in all 3 genomes, which may be responsible for the production of 14- and 18-residue peptaibols, respectively. Furthermore, the analyis revealed an identical 18-module NRPS synthase in all 3 species.

A bioinformatic strategy was used to predict the peptaibol-producing ability of *Trichoderma* green moulds by comparing their NRPS genes with those of other members of the genus. Draft genome sequences of *T. pleuroti* and *T. aggressivum*, and genomes of *T. reesei*, *T. longibrachiatum*, *T. citrinoviride*, *T. virens*, *T. harzianum* and *T. atroviride* were studied with Antibiotics and Secondary Metabolites Analysis Shell (antiSMASH) and the PKS/NRPS Analysis Web Site. Single candidate NRPS genes encoding 18-module proteins were identified in both green mould species. The deduced amino acid sequences of the putative NRPS genes of *T. pleuroti* (*nps1tp*, 62.9 kb) and *T. aggressivum* (*nps1ta*, 62.9 kb) consist of 20963 and 20979 amino acids, respectively, with an estimated molecular mass of 2.3 MDa for both. The deduced proteins contain single ketoacyl synthase and acyl transferase domains at their N-terminal, followed by modules consisting of adenylation, thiolation and condensation domains, and a thioesterase domain at the C-terminal. Prediction of incorporated amino acids based on 8-amino acid signature sequences in the adenylation domains was performed by NRPS/PKS substrate predictor and NRPSPredictor2 SVM, with the TEX1 protein of *T. virens* included as comparison. Signature sequences were identical for NPS1tp, NPS1ta and TEX1 in 7 positions. Green moulds shared the same signature sequence in 4 further modules, while 2 modules were identical between *T. aggressivum* and *T. virens*, while *T. pleuroti* had the same signature sequence as *T. virens* in module 10. Predictions by NRPS/PKS substrate predictor were in full agreement in 7 positions for both green moulds with the peptaibol sequences detected in their extracts, while NRPSPredictor2 SVM revealed further matches, suggesting that the putative

NRPS proteins are able to produce the detected peptaibols. The presence of secondary metabolism-associated genes in the genomic region downstream of *nps1ta* and *nps1tp* also suggests that the genes are operational.

Annotation of 14-residue peptaibol synthases in the 3 green-mold species along with *T. harzianum*, *T. reesei* and *T. virens* revealed that all genomes contained the 14-residue NRPSs, except from *T. harzianum* which has a 12- instead of 14-residue synthase. AntiSMASH analysis showed that these NRPSs contain acetyl transferase domain and high ratio of aminoisobutyric acid, which are specific features of peptaibol synthases. Amino acids of the binding pockets were concatenated and submitted to Clustal Omega. Based on the binding pocket sequences our hypothesis was that *T. harzianum* lost 2 modules responsible for incorporation of the amino acids into positions 5 and 6, thus we skipped the amino acids in these positions and continued with position 7. Phylogenetic analysis of these binding pockets showed high homology between *T. pleuroti* and *T. pleuroticola* as well as *T. aggressivum* and *T. virens*. In the case of *T. harzianum*, in position 7 there is an amino acid change (while *T. virens* has 2, *T. reesei* 1) but positions 4 and 8 have perfect homology in all species including *T. harzianum*, suggesting NRPS truncation, which could also influence the effect of peptaibols. Comparison of green mould and non green mould species revealed a significant change in position 7 where Leu was changed to Ala in *T. pleuroti*, *T. aggressivum* and *T. pleuroticola*.

An *in vitro* biotest method was developed for screening the bioactivity of peptaibols on mushrooms grown on agar media. Discs 5 mm in diameter deriving from the edge of freshly growing *Agaricus* or *Pleurotus* colonies were inoculated into the center of Petri plates (9 cm in diameter) and incubated at 25 °C until a colony diameter of 3 cm. Subsequently, holes 5 mm in diameter were bored into the agar 0.5 cm away from the mushroom colony and filled with 40 µl amounts from a dilution series of alamethicin (Alm) solution. After a further incubation of 1-2 d for *Pleurotus* and 1-2 weeks for *Agaricus*, eventual inhibition was visually recorded. The method proved appropriate to detect growth inhibition of mushroom mycelia by peptaibols.

We aimed to get more insight into the peptaibol production of *T. aggressivum* and *T. pleuroti*. HPLC/MS-based methods revealed the production of peptaibols closely related to hypomurocins B by *T. aggressivum*, while tripleurins representing a new group of 18-residue peptaibols were identified in *T. pleuroti*. For preparative purification of these peptaibols, strains were cultivated on malt extract agar (MEA) in 40×40 cm plates for 7 d at 25 °C. Biomass was harvested from the surface of the media and peptaibols extracted by methanol/chloroform. Crude extracts were checked for their inhibitory effects on mushroom mycelia by the *in vitro* biotest optimized with Alm. Growth inhibition of *A. bisporus* and *P. ostreatus* was observed after 3 d of incubation, but finally the mushrooms overgrew the holes. The same method was used to observe the effects of peptaibols on mushroom mycelia grown on media simulating cultivation conditions: concentrations of 100 and 50 mg/ml peptaibol extracts stopped the growth of *P. ostreatus* with inhibition zones of 23 mm and 17 mm, respectively. In further experiments, Petri-plates were filled with MEA, the surface of the media covered by cellophane and centrally inoculated with *Trichoderma*. The cellophane was removed after 3 d at 20°C, *A. bisporus* or *P. ostreatus* inoculated to the center of the plates, and the diameters of their colonies measured after 5 d at 20°C. *P. ostreatus* showed 23 and 42% decrease in growth on the surface of media with previous growth of *T. aggressivum* and *T. pleuroti*, respectively, suggesting that peptaibols have inhibitory effects on the host mushrooms.

Accelerated Molecular Dynamics (aMD), which can elucidate the dynamic path from the unfolded state to the near-native state, “flattened” by introducing a non-negative boost to the potential, was tested with the *Trichoderma* peptaibol Alm F30/3. Three consecutive simulations of ~900 ns each were carried out where N-terminal folding could be observed within the first 100 ns, while C-terminal folding could only be achieved almost after 800 ns. It took ~1 microseconds to attain the near-native conformation with stronger potential boost,

which may take several microseconds worth of classical MD to produce the same results. The Alm F30/3 hexamer channel was also simulated in an *E. coli* mimicking membrane under an external electric field that correlates with previous experiments. The results confirmed that aMD simulation techniques can elucidate peptaibol structures and their folding dynamics.

The 3D structure of TPNXIIc, an 18-mer peptaibol from the group of tripleurins produced by *T. pleuroti* was studied to understand its modes of action. Non-standard residues were parameterized by Restrained Electrostatic Potential charge. aMD was used to simulate the unfolded peptide in water and chloroform solvents, in which the peptide showed different folding behavior. The largest low-energy cluster obtained from water system shows continuous β-turns, which form a β-bend ribbon spiral at the N-terminal and a 3_{10}/α-helical continuous structure at the C-terminal. In chloroform, a higher propensity to form γ-turn is observed which renders the backbone in a bent state comparable to a β-hairpin.

Peptaibol profiles of a *T. longibrachiatum* and a *T. ghanense* strains from mushroom compost, and a *T. capillare* strain from the wall of a mushroom growing cellar were determined and compared with further 14 related species from clade Longibrachiatum of the genus *Trichoderma*. Besides longibrachins, trilongins and hypophellins, the 3 strains also produced yet undescribed peptaibols, with the most new sequences identified in *T. ghanense*.

The image analysis-based method developed previously for the evaluation of the aggressivity of *Trichoderma* strains towards *P. ostreatus* was adopted and optimized to *A. bisporus*. For the *in vitro* confrontation assays, special media, simulating the conditions of mushroom cultivation were developed and optimized. Mycelial plugs (5 mm in diameter) cut from the actively growing edge of *Agaricus* or *Pleurotus* colonies were inoculated to 1.5 cm distance from the center of Petri-dishes (9 cm in diameter) containing the corresponding medium and incubated at 25 °C. After reaching a colony diameter of 1 cm (1-2 d for *Pleurotus*, 3-4 d for *Agaricus*), the examined *Trichoderma* isolates were inoculated 3 cm away from the center of the mushroom colony using the same method. The plates were incubated for further 3-4 d at 25 °C. After the incubation period the plates were photographed and aggressivity indices (AI) were calculated according to the formula: AI = (area of the *Trichoderma* colony / total area covered by the *Trichoderma* and the mushroom colony) × 100. Interactions between *T. aggressivum*, *T. pleuroti*, *T. pleuroticola*, *T. harzianum*, *T. decipiens*, and the cultivated mushrooms *A. bisporus* and *P. ostreatus* were studied. All examined *Trichoderma* species proved to be aggressive towards both *A. bisporus* (AI= 83.2-100) and *P. ostreatus* (AI= 30.54-100), suggesting their potential to impair both mushrooms. Gfp-labelled *Trichoderma* green mould strains were also confronted with the mushrooms on media covered by cellophane membrane. After 2 days of co-culturing, 1×1 cm pieces were cut from the cellophane in the confrontation zone of *Trichoderma* and the mushroom and examined by fluorescence microscopy. Differences between autofluorescence of mushrooms/parental *Trichoderma* strains and fluorescence of gfp-labelled derivatives could be clearly detected, but no fluorescence was observed following growth on media amended with *Pleurotus* substrate or *Agaricus* compost, suggesting that gfp-labelled strains may not be applicable for monitoring green mould infections in mushroom cultivation. Gfp-labelled *T. aggressivum* and *T. pleuroti* showed aggressivity towards *P. ostreatus* similar to that of their parental strains, while lower AI values were obtained for *T. pleuroticola*. All gfp-labelled strains showed reduced growth on the medium simulating *A. bisporus* cultivation: no contact was observed between them and the host mushroom. Coiling of *Trichoderma* species around hyphae of the host mushrooms could not be observed, so it cannot be ruled out that *Trichoderma* green moulds apply mechanisms other than mycoparasitism to attack their hosts.

Confrontation assays were performed with inoculation of *T. pleuroti* against oyster mushroom or itself on minimal medium covered by cellophane. Self-confrontations of *Trichoderma* were performed in order to filter out gene expression changes related with general

recognition processes. Fungal biomasses were collected at 2 different points of time, 1 cm before contact and 48 hours after contact. RNA isolation was performed by the PrepEase RNA Spin Kit. Quality and quantity of RNA were checked by an Agilent 2100 Bioanalyzer, RNA Integrity Number above 7 was used for the library preparation only. Good quality of total RNA was ensured by MagJET mRNA Enrichment Kit. Transcriptome libraries were constructed by the Ion Total RNA Seq Kit v2 and measured by Agilent 2100 Bioanalyzer to assay the molar concentration and size distribution. Emulsion PCR was performed with Ion OneTouch 2 and the sequencing with an Ion PGM sequencer. Quality check and demultiplexing of reads were done by Torrent Suite. Reads were converted to fastq files and aligned to the reference genome using Bowtie2 and TopHat. Differential gene expression was determined by Cuffdiff following the assembly of aligned reads by Cufflinks. Finally the transcripts for gene expressions log2 fold change values above 5 upstream and under -5 downstream were filtered to focus on the highest differences. The filtered gene set was submitted to Standalone BLASTx to identify the functions of the genes. Search was performed against non-redundant protein database using an E-value cutoff 0.001, word size of 6 and HSP Length Cutoff 33. Identification of highly differentially expressed genes resulted in 11 unique upregulated genes with predicted function: a multidrog resistance (MDR), a carbohydrate-binding, an SSCR, a ubiquitin-conjugating, a proteinase inhibitor, an esterase, a subtilisin, a cytochrome, a quinoprotein amino dehydrogenase, a ubiqutin-binding and an N-terminal fungal transcription factor were determined. The most extensively upregulated gene is the MDR protein which may play a role in the defence mechanism against secondary metabolites secreted by the mushroom. Seven uniqe downregulated genes with known functions could also be identified: a formaldehyde-activating, a 2,5-didehydrogluconate reductase, a purine and uridine phosphorylase, a transmembrane amino acid transporter, a deoxyribose-phosphate aldolase, an ATPase domain containing and a glycosyltransferase protein. Primers were designed by Primer3 for the gene sequences showing high expression differences. In order to measure gene expression for the putative peptaibol synthases, the acetyl transferase domains were collected from the genomes and searched for 100% homologous sesquences. Peptaibol synthase-specific primers for qPCR were designed based on the homologous nucleotide sequences using Unipro Ugene manually, thus 1 universal primer can be used for the comparison of the 3 species.

During the project period, a green mould epidemic outbreak occurred at a Hungarian mushroom farm in the cultivation of both *A. bisporus* and *P. ostreatus*. Within the frames of an epidemiological survey, we collected samples at the affected farm from compost ingredients, compost sterilization tunnels, 1st, 2nd and 3rd phase compost, as well as from cultivation batches of *Agaricus* and *Pleurotus* affected by green mould symptoms. A total of 15 *Trichoderma* strains were isolated from the collected samples. Among the compost ingredients only peat samples contained *Trichoderma*. No *Trichoderma* growth occurred on *Trichoderma*-selective medium in the case of samples collected from growing houses without symptoms of green mould, while intensive colonization of the medium was observed following inoculation with green mould-affected *Agaricus* compost and casing soil, as well as *Pleurotus* substrate. The isolated strains were pure cultured and identified by ITS barcoding. Various *Trichoderma* species (*T. harzianum*, *T. virens*, *T. atroviride* and *T. ghanense*) were detected in peat samples, while the dominance of *T. aggressivum* was found in green mould-affected *Agaricus* compost and casing soil. From green mould-affected *Pleurotus* substrate, *T. aggressivum* was isolated together with *T. pleuroti*. This is the first observation about the occurrence of the *Agaricus* green mould agent *T. aggressivum* in the cultivation of oyster mushroom. Furthermore, the *T. aggressivum* strains isolated during the outbreak were identified as representatives of the North-American green mould biotype *T. aggressivum* f. *aggressivum*, and their aggressiveness was confirmed by Koch's postulates in artificially infected *Agaricus* cultivation minibags. These are the first data about the appearance of *T. aggressivum* f. *aggressivum* in Hungary and Europe.

A white mould epidemic outbreak resulting in serious crop losses also occurred at 2 Hungarian *Agaricus* farms: the causal agent was identified as *T. decipiens*, a species which has not yet been reported from the cultivation of *A. bisporus* before.

A series of *Trichoderma* strains previously isolated from mushroom growing substrates and initially identified as *T. harzianum* species complex members based on ITS barcoding were subjected to the amplification, partial sequencing and sequence analysis of the *tef1*, calmodulin (*cal*) and RNA-polymerase II subunit (*rpb2*) genes. Besides *T. harzianum sensu stricto* (white button mushroom compost, Hungary, Croatia; oyster mushroom cultivation substrate, North Macedonia), other species of the Harzianum clade – *T. guizhouense* (oyster mushroom cultivation substrate, Serbia, Croatia), *T. atrobrunneum* (oyster mushroom cultivation substrate, North Macedonia; shiitake cultivation substrate, Serbia), *T. simmonsii* (oyster mushroom cultivation substrate, North Macedonia, Serbia; shiitake cultivation substrate, Hungary, Serbia), *T. afroharzianum* (oyster mushroom cultivation substrate, North Macedonia, Serbia, Spain) and *T. pollinicola* (shiitake cultivation substrate, Hungary) – are also occurring in mushroom cultivation and may be responsible for green mould infections. For strains belonging to *T. longibrachiatum*, which is also frequently occurring in mushroom growing substrates, we performed multilocus phylogenetic analysis, physiological investigations by BIOLOG Phenotype Microarrays and antifungal susceptibility testing by Etest. Results supported that agricultural environments including mushroom farms may be possible sources of the emerging human fungal pathogen *T. longibrachiatum*, and we proposed the re-classification of the recently described species *T. bissettii* as *T. longibrachiatum* f. sp. *bissettii.*

The ability of *T. aggressivum* f. *europaeum* and f. *aggressivum* isolates to utilize different carbon sources was studied in plate assays (solid minimal medium, 25 ^{o}C) in comparison with *A. bisporus* strain SZMC 23395. Several carbon sources (e.g. certain sugars, amino and other acids) were found to significantly inhibit the mycelial growth of the *T. aggressivum* isolates, however, the majority of them had strong negative effect also on the growth of *A. bisporus*. The only exception was L-sorbose, which caused 65-74% inhibition of *T. aggressivum*, while having little negative effect on *A. bisporus* (5% inhibition). This compound might be considered as a potential agent to control green mould disease. Further carbon source utilization assays were performed by BIOLOG Phenotype Microarrays on *T. aggressivum* f. *aggressivum*, *T. aggressivum* f. *europaeum*, *T. decipiens* and *T. harzianum* isolates. *T. aggressivum* f. *europaeum* could be distinguished clearly from *T. aggressivum* f. *aggressivum* also on the basis of their carbon source utilization profile, however, only minor variance was detected between the different isolates. *T. decipiens* was found to have a characteristic, low growth profile compared to the rest of the examined species.

The sensitivity of *Trichoderma* strains from edible mushrooms to the fungicides prochloraz and metrafenone was determined. During a large survey of the *in vitro* antagonism of compost-derived bacterial strains, 79 *Bacillus* and 91 *Pseudomonas* isolates were tested against 2 *T. aggressivum* f. *europaeum* and 1 *T. aggressivum* f. *aggressivum* strain, resulting in the selection of 46 *Bacillus* and 29 *Pseudomonas* strains with inhibitory activity for further *in vitro* antagonism experiments performed against *Cladobotryum mycophilum*, the causal agent of the cobweb disease. The results allowed the selection of bacterial strains potentially applicable for the purposes of biological control of weed moulds in mushroom cultivation.

A 1200 liter volume Weiss Gallenkamp SGC120 standard plant growth chamber was adapted to experimental mushroom production by optimizing the environmental parameters (temperature, humidity) for the cultivation of *A. bisporus* on mushroom compost covered with casing material in commercially available compost blocks of 70×45×15 cm wrapped in polyethylene (approx. 12 kg) and 30×20×15 cm mushroom growing boxes (approx. 4.5 kg). The phase 3 compost in the products consisted of wheat straw, chicken manure, horse manure, gypsum and white hybrid *Agaricus* spawn. Two compost blocks and 3 mushroom growing

boxes (with a compost height of 15 cm for both) were covered with approx. 5 cm of black peat casing material. A cultivation experiment of 36 days was designed with temperature and humidity values set in the ranges of 17-21 °C and 85-100%, respectively, adapting the conditions of mushroom growing houses to the volume of the plant growth chamber. Compost colonization and fruiting body formation proved to be appropriate under the controlled conditions, but the compost blocks became affected by fungal infections during the 2nd harvesting period (days 34 to 36) and yielded 85% less crop than in the 1st harvesting period. The infecting moulds were identified by ITS barcoding as *Mycogone perniciosa*, the causal agent of „wet bubble", and *Acremonium* cf. *camptosporum*. Mushroom flies – identitified as *Lycoriella ingenua* (Sciaridae) by the sequence analysis of a fragment of the cytochrome oxidase I (COI) gene – also appeared, which are known to serve as vectors of mould infections. The mushroom growing boxes remained symptomless, suggesting that the casing material supplied with the mushroom compost blocks may have been the source of contamination, thus, to reduce the risk of mushroom crop losses in the plant growth chamber, sterilization of the black peat by autoclaving is recommended before casing.

Different cultivation volumes were tested in the plant growth chamber, using sterilized casing material and CACing (compost addition to casing) to establish an experimental microcompost system allowing parallel cultivation in several replicates, thereby providing a powerful tool to study the interactions of mushroom pathogenic microorganisms, cultivated mushrooms and potential biocontrol agents. A pot experiment was designed with the application of 15×15×10 cm size plastic pots. For inoculation, a bacterial strain promising for biocontrol (*Bacillus velezensis* SZMC 25431) and a green mould strain (*Trichoderma aggressivum* f. *europaeum* SZMC 1746) were selected. Eight different treatments were applied during the experiment: the casing layer was always sterile, while the compost was either living or sterilized, and in both series the casing layer was untreated, treated with *T. aggressivum* alone, treated with *B. velezensis* alone or treated with both *T. aggressivum* and *B. velezensis*. The 1st, 2nd and 3rd harvests were started on days 19, 29 and 40, respectively. The experiment was running for 45 days until the end of the 3rd harvesting period, before which the signs of green mould could be detected in the pots containing living compost inoculated with *T. aggressivum* alone or together with *B. velezensis*. No symptoms occured in pots with sterile compost infected with *Trichoderma*. The results suggested that for stable, reproducible *Trichoderma* infection, the mixing of mould inoculum into the compost is necessary. During a repeated experiment, samples taken from the differently treated casing layers after the 1st and 2nd crop wave, as well as at the end of the experiment were subjected to total RNA isolation for transcriptomic analysis. The *B. velezensis* strain slightly increased the crop amount, and although it has not provided complete protection against the green mould, the symptoms were milder in pots treated with *B. velezensis* and infected with *T. aggressivum*. Based on the results, an experiment was set up in a mushroom farm, where 2 out of 3, 5-storeyed growing shelves were treated with 2 different concentrations of *B. velezensis*, while the 3rd shelf was left untreated as the control. The treatment was administered by spraying on the top of the casing layer covering the freshly filled-in compost. The treatment decreased the occurrence and damage of dry bubble disease caused by *Lecanicillium fungicola*.

Experiments were carried out also with fly traps for thinning mushroom fly populations acting as vectors of weed moulds in mushroom growing houses. Various plant essential oils (citrus, thyme, citronella cinnamon, clove) and 4 steroid compounds were dissolved in water or beer and filled in traps made of plastic Falcon tubes. Citronella dissolved in water showed repellent properties, while dissolved in beer it proved to be an attractant. Certain steroid compounds were very efficient attractants even at very low concentrations.

The publication of the project results will be continued in the post-project period with at least 2 additional journal articles and a book chapter indicating the identifier of the project.