Szent István University

Potential of *Trichoderma* species and nematode-trapping fungi to control plant-parasitic nematodes: *in vitro* confrontation and gene expression assays using *Caenorhabditis elegans* model system

PhD Thesis

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1. BACKGROUND AND OBJECTIVES

Plant-parasitic nematodes have substantial impact on human welfare and economy either by attacking root systems, stems, buds or by acting as vectors of plant viruses. Despite the propensity of nematicides to be lethal to a broad range of organisms, and to induce the development of resistant strains among nematodes, they are still widely used to control plant-parasitic nematodes.

Though numerous potential biocontrol agents (BCAs) have been reported that may have the ability to replace pesticides with environmentally safer methods only a few have been commercialized. Among them *Trichoderma* species have long been recognized and highly valued as BCAs of foliar and soil borne diseases. They possess complex chitino- and proteolytic system which makes them excellent competitors in the soil environment and there are also reports on successful attempts to control plant-parasitic nematodes with them. Among nematophagous fungi, nematode-trapping fungi are also attracting scientific interest as they have the potential to be used as BCAs against plant-parasitic nematodes. Applying certain strains of *Trichoderma* and nematode-trapping fungi in one preparation can lead to enhanced biocontrol effect. However, the combination of more than one species or isolate is not always sufficient due to incompatibility or antagonistic properties.

While so far little is known about the roles of chitinase and peptidase encoding genes during nematode egg-parasitism of certain *Trichoderma* species, in a series of recent studies, they were characterized during several simulated biocontrol processes (e.g. *in vitro* confrontation against fungal plant pathogens and different nutrient stress conditions such as carbon starvation, glucose, chitin and cell walls of different fungal plant pathogens

as carbon source). We made simultaneous analysis of gene expression patterns during nematode egg-parasitism and comparison with other biocontrol processes that may reveal functional and regulation diversities among certain chitinase and peptidase encoding genes. These results can contribute to a better understanding of the complex host-pathogen interactions and provide background for improvement of *Trichoderma* strains to be used as agents for the biological control of parasitic nematodes.

OBJECTIVES

1. To elucidate the combinability of nematode-trapping fungi and *Trichoderma* strains.
2. To create *in vitro* experimental system for the selection of the most effective *Trichoderma* strains and for revealing the molecular background of the host-pathogen interactions.
3. Transcriptome analysis of chitinase and peptidase encoding genes of the most effective *Trichoderma* strain.
4. Comparison of gene expression patterns during nematode egg-parasitism and other biocontrol processes of the most effective *Trichoderma* strain in order to elucidate functional and regulational diversities among certain chitinase and peptidase encoding genes.

2. MATERIALS AND METHODS

2.1. Media and culture conditions of nematode-trapping fungi

Nematode-trapping fungi *Arthrobotrys oligospora*, *A. tortor*, *Monacrosporium haptotylum* and *M. cionopagum* are originated from international culture collections. They were cultured on corn meal agar (CMA; Oxoid) and soy peptone plates (SP), respectively.

2.2. Media and culture conditions of *Trichoderma* strains

The strains of *Trichoderma harzianum*, *T. virens*, *T. atroviride*, *T. rossicum* and *T. tomentosum* were obtained from the Microbiological Collection of the University of Szeged, Hungary and maintained on potato dextrose agar (PDA; Oxoid). For the interaction of *Trichoderma* and *C. elegans* eggs, *Trichoderma* strains were grown on cellophane-covered synthetic medium (SM) at 25 °C in dark.

2.3. Maintenance of *Caenorhabditis elegans*

Wild type N2 (Bristol) *C. elegans* was grown monoxenically using *Escherichia coli* strain OP50 as a food source and maintained on Nematode Growth Medium (NGM).

2.4. Growth rate measurement of nematode-trapping fungi and *Trichoderma* strains

Growth rate of each monocultural fungus were observed on CMA medium by measuring radial growth daily over a 5-day period. Each experiment was replicated three times.

2.5. Direct confrontation assay of nematode-trapping fungi and *Trichoderma* strains

To estimate the compatibility of 18 strains of five *Trichoderma* species and six strains of four species, nematode-trapping fungi confrontation assays were performed on SM agar plates. Agar plugs were removed from the

plates on which the various fungi were maintained as follows: plugs were cut from the growing edge of three-day old colonies of each fungus and were placed on the opposite sides of the SM agar plates. In order to facilitate microscopic observation agar plugs were also placed on cellophane covered SM agar plates, too. Dual cultures were allowed to grow until they physically contacted one another at 25 °C in a dark chamber.

2.6. *Assay of egg-antagonism*

C. elegans eggs were prepared from gravid hermaphrodites according to the sodium hydroxide, sodium hypochlorite method (Eisenmann, 2005). Each *Trichoderma* strain was pre-cultured on SM medium for 3 days and then received approximately $3x10^2$ eggs. The Petri dishes were sealed and incubated at 25 °C in the dark until examination. Each experiment was replicated three times. Axenically plated eggs of *C. elegans* (in three technical repetitions) were used to generate EPI numbers. EPI was calculated 11 hpi by dividing the number of hatched larvae in the presence of *Trichoderma* by the number of hatched larvae in the axenically plated control. The hatched larvae were counted in gridded Petri dishes (0.25 mm^2 per square, 60mm DIA). The obtained results were analysed by one-way ANOVA statistic using Microsoft Excel Analysis ToolPak add-in.

2.7. Microscopic observation

The interaction zones of nematode-trapping fungi and *Trichoderma* strains were stained with calcofluor white following the manufacturer's recommendations (Sigma), then microphotographed using a Nikon Eclipse E80i microscope without fixation. Calcofluor white staining was used also to detect hyphal attachment of *Trichoderma* to the *C. elegans* egg surface.

2.8. Interaction of *C. elegans* eggs and *T. harzianum* (SZMC 1647) for gene-expression experiment

C. elegans eggs were prepared from gravid hermaphrodites according to the sodium hydroxide, sodium hypochlorite method (Eisenmann, 2005). *T. harzianum* (SZMC 1647) was grown on cellophane-covered SM plates for three days and then $\approx 3x10^2$ eggs were placed near the edge of the mycelia in a 5 mm wide zone on each plate. Covering the *ex utero* development of *C. elegans* eggs (approximately 9-11 h); nearby 5 mm zones of egg-induced mycelia were collected at seven time points (0, 1, 3, 5, 7, 9 and 11 h post-inoculation, hpi). Each time sample was pooled from thirty interactions, flash-frozen in liquid nitrogen, and stored at -80 °C until RNA extraction. To reconfirm egg-induced gene expression, a set of control experiment (without eggs) was also conducted.

2.9. RNA extraction, primer design, real-time PCR, and data analysis

Total RNA was extracted from 25 mg of mycelial mats (fresh weight) according to the method described by Stiekema et al. (1988). After DNase treatment (Promega RQ1 RNase-free DNase), RNA concentration and quality were determined using an Agilent Bioanalyzer 2100 instrument (Agilent RNA 6000 Nano reagent kit). Samples which had an RNA integrity number (RIN) greater than or equal to 7.0 were reverse transcribed into single-stranded cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's recommendations. Gene-specific oligonucleotide primers were designed by using the web-based primer design service (Primer3). Master mixes and PCR reaction plates were prepared by epMotion 5070 automated pipetting system (Eppendorf). PCR reactions were performed with a StepOne™ Real-Time PCR system (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) to monitor gene-specific amplicon synthesis.

The following thermal profile was used for all PCRs: an initial denaturation step at 95 ºC for 10 min, followed by 45 cycles at 95 ºC for 15 s, 58 ºC for 20 s, and 72 ºC for 20 s, and a final extension at 72 ºC for 2 min. Reactions were set up in triplicate, including a control with no template. The specificity of each amplification process was confirmed by melting curves. The qRT-PCR data were analyzed using relative quantification according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) integrated into the ABI software of the StepOne Real-Time PCR instrument. Applied standard (actin) was stably expressed at the same level throughout all reaction and its expression was not influenced by the experimental conditions (e.g. addition of eggs, physiological state of the colonies). The degree of change in transcript abundance of peptidase-encoding genes was compared by using the difference in $\log_2$ values between steady-state level of the peptidase transcripts relative to 0 hpi as the calibrator sample, and each of the other six time points (1, 3, 5, 7, 9, 11 hpi). To reconfirm egg-induced gene expression, a set of control experiment (without eggs) was also conducted. Each qPCR experiment was designed and performed according to the MIQE (Minimum Information for Publication of qPCR Experiments) guide recommendation (Bustin et al., 2009).

3. RESULTS

3.1. In vitro evaluation for combinability of potential BCAs

In order to reveal the combinability of the potential candidates, six strains of four nematode-trapping fungi were tested *in vitro* against eighteen strains of five *Trichoderma* species. First, monoculture growth rate tests and dual confrontation assays were performed to evaluate the most elemental attributes of the two species. Even though *in vitro* growth rates do not fully represent growth rates in nature, they are informative about the behavior of these organisms.

A comparison of colony radius in monoculture revealed that *Trichoderma* species grow more rapidly than nematode-trapping fungi. *T. harzianum* SZMC 1647 and *T. tomentosum* SZMC 1610 represents the maximum and minimum growth rate values respectively, however the growth rate of all studied *Trichoderma* has been generally similar. *M. haptotylum* CBS 200.50 demonstrated the highest growth rate among nematophagous fungi but produced a smaller colony radius than the slowest-growing *T. tomentosum*. Of all candidates, *M. haptotylum* CBS 220.54 demonstrated the lowest growth rate. Henceforth, those strains of *Trichoderma* and nematophagous fungi were tested in confrontation assays which did not differ significantly in their monoculture growth potential since significant differences in *in vitro* growth rate may exclude the feasibility of combining different species.

The evaluation of 90 direct confrontation assays (*Trichoderma* spp. *vs.* nematode-trapping fungi) indicated that *Trichoderma* isolates antagonize nematode-trapping fungi in 15 % of the cases by antibiosis and in 80 % of the cases by rapid overgrowth. In dual culture, *T. harzianum* strains (SZMC 1600, 1647, 1677 and 2636) and *M. cionopagum* (CBS 228.52) have shown nearly identical growth rate. Even three weeks after inoculation, *T.*

harzianum strains (1600, 1647, 1677 and 2636) were unable to occupy *M. cionopagum* (CBS 228.52). Interestingly, coiling around the hyphae of nematode-trapping fungi, which is a typical form of mycoparasitism in *Trichoderma*, was not observable in these assays. The fungal strains rather grew over or followed each other's hyphae in a straight course. Attachments of hyphae were only seldom observable, especially when one of the partners was *A. oligospora*. Interestingly, *A. oligospora* can attack other fungi by coiling around the host hyphae, which results in disintegration of the host cell cytoplasm without penetration of the host (Nordbring-Hertz et al., 2006). Because of the nonselective staining of the interacting hyphae, in these cases, it was not determinable which partner produced hyphal attachment. In either way, if one partner is able to parasitize the hyphae of the other candidate the two fungi cannot be used together in one preparation.

3.2. The effect of *Trichoderma* species on eggs of *C. elegans*

The microscopic observation of egg-fungus interactions revealed clear evidence that all characteristic events of egg-parasitism (*ie*. coiling hyphae around eggs, formation of appressorium-like structures, trophic hyphae growing inside the eggs) occurred in a time period of 11 hours.

Antagonism against eggs of *C. elegans* by each *Trichoderma* strain was evaluated using EPI numbers (Egg-Parasitic Index) which was calculated 11 hpi by dividing the number of hatched larvae in the presence of *Trichoderma* by the number of hatched larvae in the axenically plated control. EPI numbers varied among species and strains between 0.26-0.70. Deviations of each strain from control were proven statistically significant ($P \leq 0.05$). Within *T. harzianum* and *T. atroviride* species EPI did not differ significantly but strains of *T. virens* showed significantly different EPI

values in several cases ($P \leq 0.5$). EPI of *T. virens* and *T. atroviride* strains were lowest, ranging from 0.31 to 0.48 and from 0.68 to 0.71, respectively. EPI of *T. tomentosum* and *T. rossicum* was 0.40. *T. harzianum* strains parasitized the most eggs during the time-course of the examination (EPI: 0.26-0.32). These data have shown that *T. harzianum* strains have the innate ability to parasitize nematode eggs. One of the most effective strains of *T. harzianum* (SZMC 1647; EPI: 0.26) was selected to determine the gene expression patterns of the key chitino- and proteolytic enzymes in the process of egg-parasitism.

3.3. Transcription activity of chitino- and protelytic enzyme encoding genes of *T. harzianum* during *C. elegans* egg-parasitism

3.3.1. Transcription activity of endochitinase encoding genes

The otherwise weakly expressed *chi18-12* during mycoparasitism did not varied significantly in the untreated samples but was strongly transcribed during the nematode egg-fungus interaction. In the treated samples, transcript abundance of *chi18-12* increased dramatically at the beginning and then gradually increased until 5 hpi. After the peak at 5 hpi, *chi18-12* gene expression dropped to almost zero which was followed by a gradual increase until the end of the interaction.

As for *chi18-5*, its gene expression was significantly up-regulated first at 5 hpi and then dropped to base level. This was followed by a gradual increase until the end of the interaction. On the contrary, according to Zeilinger et al., *chi18-5* is involved in the very early stages during mycoparasitic process which precedes physical contact with the host.

Transcript levels of the two chitinases indicated that the expression values of *chi18-12* were significantly higher than those of *chi18-5* except at 7 hpi and *chi18-12* was upregulated in the early stages of the egg-parasitic process, 3-4 hours before *chi18-5* induction. In general, the expression

patterns of both chitinase genes revealed that the two genes had parallel and probably complementary modes of action during nematode egg infection process.

3.3.2. Transcription activity of serine endopeptidase encoding genes

To date, only a trypsin-like acidic serine peptidase and an alkaline serine peptidase of *T. harzianum* encoded by *pra1* and *prb1* genes, respectively, have been known for their nematicidal effect (Sharon et al., 2001; Suárez et al., 2004).

During egg-parasitic interaction, expression pattern of *pra1* revealed significant up-regulation from 5 hpi. Our finding is in a good agreement with the study of Suárez et al. (2004) in which *pra1* was upregulated as early as after 4 h, when *T. harzianum* was grown in minimal medium supplemented with chitin or fungal cell walls. At the following two time points (9 and 24 h) they found dramatic decrease in *pra1* gene activity. On the contrary, during egg parasitism, the *pra1* mRNA abundance remained stable at a relatively high level at 7 and 9 hpi. Even a higher expression level of *pra1* was detected at the end of the *ex utero* development of the *C. elegans* eggs.

As for the untreated samples, the transcription level of *prb1* was very high in the fifth, ninth and the eleventh hours of the experiment relative to the zero time point. On the contrary, during egg-parasitism, it was mostly downregulated or there was no significant variation in the levels of its corresponding messenger RNA and exhibited minor up-regulation relative to the zero time point only at 5 hpi. The overall low gene expression of *prb1* suggests that it may have no function in the process of nematode egg-parasitism.

Another member of the subtilisin subfamily S8A, a serine endopeptidase encoded by the *p8048* gene was characteristically but weakly triggered by the presence of nematode eggs at each time point. The mRNA abundance of *p8048* varied mainly within a narrow range, though finally a moderately higher value was reached at 11 hpi. Its non-constitutive expression profile was similar to *pra1*; however, *p8048* was manifested generally on a lower level. These results are in an agreement with the finding of Suárez et al. (2007), although, unlike during egg parasitism, fungal cell walls and chitin induced higher transcription activity of *p8048*.

The serine peptidase encoded *p5431* was constitutively transcribed at all time points in the presence of glucose, chitin and *Botrytis cinerea* cell wall as well as in the absence of carbon and nitrogen source (Suárez et al., 2007). Interestingly, *p5431* was significantly downregulated at each time point relative to reference sample (0 hpi) and the untreated samples during nematode egg-parasitism. It may have minor or no rules in nematode egg-parasitism.

The subtilisin-like serine endopeptidase-encoding gene *SS10* was also downregulated throughout the fungus-egg interaction and in the untreated samples, too; however Yan and Qian (2009) reported in a previous study that *SS10* seemed to strongly correspond to inducer stimuli such as *B. cinerea* fungal cell wall and chitin. It is worth pointing out that gene expression of *p7129*, a homologue of *SS10* in the study of Suárez et al. (2007) was undetectable under all simulated biocontrol conditions such as growth on five different fungal cell walls (not including *B. cinerea*) and on chitin, which is quite similar to our results. Taking into account the overall down-regulation of *p5431* and *SS10* we conclude that these genes may have less significant roles or no functions at all in the process of nematode egg-parasitism.

Transcripts of the aorsin serine peptidase-encoding gene *p5216*, a member of the family S53 were found not to respond to any of the previously tested biocontrol related conditions (Suárez et al., 2007); however *p5216* revealed the highest transcript abundance among all studied peptidase-encoding genes in the presence of *C. elegans* eggs. It was nearly two times upregulated in the early stages of interaction (at 1 hpi and 3 hpi) and then further increased at 5 hpi. Henceforth, gene expression remained around the same level until the end of the *ex utero* development of *C. elegans*. These findings highlight a new possible biological function of *Trichoderma* aorsin serine peptidase, suggesting that it may have an essential role in nematode egg-parasitism.

3.3.3. Transcription activity of aspartyl endopeptidase encoding genes

In a previous study, the examination of mRNA and protein abundance of the corresponding aspartyl peptidase P6281 clearly indicated that it was induced by different fungal cell walls (Suárez et al., 2005). Comparison of fungal cell wall- and nematode egg-induced expression profiles reveals similarities and differences regarding that in both case an early accumulation of mRNA was observed; however during egg-parasitism, a second peak was detected after a temporary decay of the gene expression. Comparing amplitudes of *p6281* to *pra1* and *p8048*, it is striking that they are displaying similar expression profiles during egg-parasitism from the fifth hour of the interaction with the only exception that *p6281* has larger deflection.

Suárez et al. (2007) reported that the putative aspartyl peptidase P9438 was probably not a secreted or membrane-associated protein and was late-responding (24 h) during each biocontrol-related condition except from chitin, on which it was early regulated (4 h) at a hardly detectable level.

Interestingly, during nematode-egg parasitism, it showed up-regulation at a moderately high level relative to the reference sample (zero time point) and to the untreated samples at each time point. It may have a moderate role in the egg-parasitic process.

The gene expression of *p7959* did not vary greatly and remained at a low level during egg-parasitism; however, during simulated biological processes, it showed transcription activity on different levels at each time point (Suárez et al., 2007). It seems that this gene left unaffected by the presence of nematode eggs.

In a good agreement with the earlier findings of Suárez et al. (2007), *p1324* was not significantly regulated in the present study.

Nematode eggs were also not able to trigger significant up-regulation of *SA76*, although it was successfully induced by different fungal cell walls and chitin (Yan and Qian, 2007).

3.3.4. Transcription activity of metalloendopeptidase encoding genes

The expression pattern of the aminopeptidase *p2920* is similar to the aspartyl peptidase *p7959*, except that the former has higher values. Under other simulated biocontrol conditions, *p2920* and *p7959* showed similar expression patterns, too; however, in this case *p2920* possessed lower values (Suárez et al., 2007). The upregulation of *p2920*, however, cannot be clearly explained by the presence of nematode eggs. Although it was underregulated relative to zero time point, it was active in the untreated samples, too. Presumably, it has minor or no rules at all in nematode egg-parasitism.

It was previously reported that neither the presence of fungal cell walls nor the absence of carbon/nitrogen sources nor addition of glucose were able to induce *p7455* expression. It showed a slight up-regulation only on

chitin (Suárez et al., 2007). On the contrary, *p7455* was characteristically transcribed during nematode egg-parasitism from 5 hpi, showing a unique time-course pattern among the presently examined peptidases. It was upregulated first at the beginning which was followed by a temporary down-regulation at 3 hpi and then its transcript abundance grew gradually reaching the maximum level at 11 hpi. According to these results, it has role in the nematode egg-parasitism of *T. harzianum.*

3.3.5. Simultaneous analysis of chitino- and proteolytic enzyme encoding gene expressions

The endochitinase-encoding gene *chi18-12* was the most intensely responding gene during the early phase of the nematode egg-parasitism (between 1 and 3 hpi). Parallel to this, the activity of the aorsin serine and aspartyl peptidase-encoding genes *p5216* and *p9438*, respectively, showed also significant increase, though their expressional changes were less considerable than those of *chi18-12*. Although the underlying causes are not fully understood, the expression of several genes become apparent for the first time on a significant level at 5 hpi (e.g. *chi18-5*, *pra1*, *prb1*, *p6281*), while others exposed further improvement (*p5216*, *p7455*) at this time point. In the seventh hour of the interaction, the amplitudes of endochitinase, aspartyl peptidase and alkaline serine peptidase-encoding genes *chi18-12*, *p6281* and *prb1*, respectively, showed dramatically decreased expression values. In contrast, the mRNA abundance of aminopeptidase *p2920* and aspartyl peptidase *p7959* was greater at 7 hpi than at 5 hpi and subsequently they exhibited gradually declining tendency. Nevertheless, from the seventh hour, the transcription of *chi18-12* and *p6281* increased considerably again until the end of the experiment.

Over-all, the expression patterns of genes encoding different chitino- and proteolytic enzymes revealed well-coordinated modes of action during

nematode egg-parasitism. In general, the highly transcribed *pra1*, *p6281*, *p9438*, *p7455*, *chi18-5* and the most prominently induced *p5216* and *chi18-12* clearly suggest that these co-expressed genes may play pivotal roles in the egg-parasitic process. Furthermore, it is well known that complex gene expression patterns result from dynamically interacting genetic networks. Thus it seems reasonable to conclude that endochitinase *chi18-5* and *chi18-12* along with *p8048*, *pra1* and *p6281* peptidase encoding genes, having CreA binding sites in their promoter regions and characterized by similar expression profile, can be co-regulated.

Even though not all upregulated genes of *T. harzianum* performed strong transcriptional response to nematode egg treatment (*p8048*, *p7959* and *p2920*), all detected up-regulations are important in the context that high nominal values are not always sufficient to predict the role of a gene. Small coordinated gene expression changes (fold change less than 2) in a complex pathway can have a major biological effect even if these changes are not statistically significant for individual genes.

NEW SCIENTIFIC RESULTS

1. It was revealed that the strains of *T. harzianum* can be combined with *M. cionopagum* (CBS 228.52) *in vitro* and thus they may be suitable partners to control plant-parasitic nematodes.

2. Based on the *ex utero* development of *C. elegans*, an *in vitro* model system was established for the examination of the molecular and microbiological background of the host-pathogen interaction.

3. *In vitro* comparison of eighteen strains of five *Trichoderma* species revealed that *T. harzianum* strains possessed the strongest egg-parasitic ability.

4. Quantitative Real Time PCR analyses of one the most effective egg-parasitic *T. harzianum* strain (SZMC 1647) revealed that two serine peptidase (*p5216*, *pra1*), two aspartyl peptidase (*p6281*, *p9438*), a metallo-endopeptidase (*p7455*) and two endochitinase (*chi18-5*, *chi18-12*) probably possess crucial role in the egg-parasitic process.

5. Based on intensive gene expression profiling and data comparison with published scientific literature, similarities (e.g. *p1324*) as well as characteristic differences (e.g. *p5216*, *p7455*, *p9438*, *p5431*, *prb1*, *SS10* and *SA76*) have been revealed between egg-parasitism and other biocontrol-related processes of *T. harzianum*.

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PUBLICATIONS

I. Publications related to the thesis:

Articles (English):

Szabó M., Csepregi K., Gálber M., Virányi F., Fekete C. 2012. Control plant-parasitic nematodes with *Trichoderma* species and nematode-trapping fungi: The role of chi18-5 and chi18-12 genes in nematode egg-parasitism. Biological Control 63, 121-128. (IF: 1,92)

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