Arboricultural Journal 2008, Vol. 31, pp. 227–248

Printed in Great Britain

IN VITRO SCREENING OF AN ANTAGONISTIC *TRICHODERMA* STRAIN AGAINST WOOD DECAY FUNGI

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The objective of the *in vitro* studies was to identify a *Trichoderma* strain with a high antagonistic potential against the basidiomycetes *Ganoderma adspersum, Ganoderma lipsiense, Inonotus hispidus, Polyporus squamosus* and the ascomycete *Kretzschmaria deusta*. For this purpose dual culture and interaction tests in wood blocks as well as investigations on fungal growth and germination behavior of conidia under different conditions were performed. Hyphal interactions were observed by scanning electron microscopy (SEM). The effect of *Trichoderma* spp. on wood colonization and degradation of wood decay fungi were quantitatively analyzed by means of dry weight loss measurements of wood and qualitatively by histological studies. The different *Trichoderma* species all showed an antagonistic potential against wood decay fungi in the *in vitro* studies. However, significant differences between the species and strains were found ($P<0.001$). *Trichoderma atroviride* (T-15603.1) showed the highest competitive activity against most wood decay fungi. An influence of physical and chemical parameters, in particular temperature and water potential on growth and germination behavior of conidia was evident. The species of wood decay fungi showed significant differences in their sensitivity when challenged by *Trichoderma*. *Polyporus squamosus* showed an extensive resistance in most laboratory tests indicating that target specificity of the antagonist needs consideration.

Introduction

Species of the genus *Trichoderma* are ubiquitous in the environment and especially in the soil. Since WEINDLING (1932) recognized the antagonistic effect of *Trichoderma* species against plant pathogens, several species of *Trichoderma* have been extensively studied as biological control agents against fungal pathogens (CHET, 1990; CHET *et al*., 1998; HOWELL, 1998). The demand for alternatives to chemical control of plant pathogens has

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become stronger owing to concerns about the safety and environmental impacts of chemicals. Today *Trichoderma* species are used in a wide range of commercial applications including the biological control of plant diseases (HJELJORD & TRONSMO, 1998; HARMAN, 2006).

Characterization of the antagonistic potential of *Trichoderma* spp. is the first step in utilizing the full potential of *Trichoderma* species for specific applications. *In vitro* screening with different bioassays is an effective and rapid method for identifying strains with antagonistic potential. For the evaluation of the antagonistic potential of different *Trichoderma* species a range of mechanisms have to be considered.

- Production of antibiotic, volatile and non-volatile chemicals. These substances influence the permeability of cell membranes and result in an efflux of the cytoplasm (HOWELL, 1998).
- Mycoparasitism and excretion of lytic enzymes. The antifungal enzyme system of *Trichoderma* spp. plays an important role for detection and destroying the host cell wall (SCHIRMBÖCK *et al*., 1994).
- Competitiveness is based on rapid growth and the production of various asexual generated conidia and chlamydospores (CHET, 1990; CHET *et al*., 1998).
- The ability to promote growth and induce resistance in plants is a mechanism which has also been described for members of this genus (HARMAN, 2006).

The objective of this investigation was to evaluate the potential of different *Trichoderma* species as biocontrol agents and to identify a competitive strain that can be used for the treatment of pruning wounds of urban trees against colonization by wood decay fungi. Successful infection and colonization of pruning wounds depends on the ability to overcome host barriers in the wood and to circumvent and/or degrade phenolic compounds (SCHWARZE *et al*., 1999, SCHWARZE & FERNER, 2003). *Inonotus hispidus* and *Polyporus squamosus* are both classified as wound parasites and are able to infect and colonize small wounds (MCCRACKEN & TOOLE, 1974, SCHWARZE *et al*., 1999). The ability of *Ganoderma. adspersum* to degrade polyphenolic deposits in reaction zones was recently demonstrated by SCHWARZE & FERNER (2003).

In addition to *in vitro* studies field experiments were performed with a highly antagonistic *Trichoderma* strain to enhance and to complete the *in vitro* investigations (SCHUBERT *et al*., 2008a).

Materials and Methods

The origin of the *Trichoderma* isolates and wood decay fungi are provided in Table 1. All cultures were maintained on 2% malt extract agar (MEA)

TABLE 1. Origin of *Trichoderma* isolates and wood decay fungi used in the present study

Trichoderma	Isolate-N°	Wood decay fungi	Isolat-N°
Trichoderma atroviride Karsten	15603.1[1]	*Polyporus squamosus* (Hud.:Fr.) Fr.	291101.2[1]
Trichoderma atroviride Karsten	CBS 351.93[2]	*Ganoderma adspersum* (S. Schulz.) Donk	086699.2[1]
Trichoderma atroviride Karsten	CBS 396.92[2]	*Ganoderma lipsiense* (Batsch) Atk.	250593.1[1]
Trichoderma fasciculatum (strictipile) Bissett*	CBS 338.93[2]	*Inonotus hispidus* (Bull.:Fr.) Karsten	200792.1[1]
Trichoderma virens Miller, Giddens & Foster	CBS 126.65[2]	*Kretzschmaria deusta* (Hoffm.) P.M.D. Mar.	271098.1[1]
BINAB TF WP (*T. harzianum/T. polysporum*)	IMI 206039/40[3]		

[1] = Isolates from Forest Botany, University of Freiburg
[2] = Isolates from Centraalbureau voor Schimmelcultures – Netherland
[3] = BINAB Bio-Innovation AB, Sweden
* = *T. fasciculatum* synonym *T. strictipile* (DRUZHININA & KUBICEK, 2005)

at 4(±1)°C. For further studies Petri dishes containing the respective media were inoculated with 0.5cm diameter agar plug, cut from the growing edge of colonies of the isolates and incubated in the dark at 25(±1)°C and 70% relative humidity.

Bioassays for growth and germination rate

The effect of temperature (5, 10, 15, 25, 30°C) and water activity (a_w 0.998, 0.955, 0.892) on the growth were detected on two different media types, 2% malt extract agar (MEA) and a modified low nutrient medium (LNA) (HUTTERMANN & VOLGER, 1973 as cited in FREITAG, 1989). The LNA- medium was selected because of its low C:N ratio which is more representative for the nutritional status of wood (SRINIVASAN *et al*., 1992). One litre contained H_2O: L-asparagine, 0.013g; KH_2PO_4, 1g; $MgSO_4$, 0.3g; KCL, 0.5g; $FeSO_4$, 0.01g; $MnSO_4$ $4H_2O$, 0.008g; $ZnSO_4$ $6H_2O$, 0.002g; $CaNO_3$ $4H_2O$, 0.05g; $CuSO_4$, 0.002g; NH_4NO_3, 0.008g; D-glucose, 5g; and agar, 10g.

All Petri dishes (90mm) were inoculated centrally with one 5mm disc of the respective *Trichoderma* isolate taken from the margin of actively growing cultures and incubated at 25(±1)°C and 70% relative humidity. For each experimental treatment (agar type, a_w and temperature) 3 replicates were performed. The growth rate was determined after 24h (mm d^{-1}) by colony diameter measurements, carried out along two perpendicular axes. The water activity of the substrate was controlled by the addition of appropriate weights of the non-ionic solute glycerol prior to autoclaving (DALLYN, 1978).

For determination of the germination rate under the specific conditions mentioned above a slight nutrient agar (SNA) was used (NIRENBERG, 1981) which contained H_2O: KH_2PO_4, 1g; KNO_3, 1g; $MgSO_4$, 0.5g; KCL, 0.5g; D-glucose, 0.2g; saccharose 0.2g; and agar, 17g per liter. After extracting agar plugs from the growth media a direct observation of conidial behaviour under the light microscope was possible after 6h, 16h, 24h and 48h. To obtain defined conidial suspensions, cultures were flooded with sterile water and filtered twice. Conidia were pelleted by centrifugation (300 rev min^{-1}) and resuspended in sterile distilled water to eliminate leached metabolites and nutrients (NAÁR & KECSKÉS, 1998). Concentrations of the conidial suspensions were determined and adjusted to approx. 10^5 cfu per ml.

Inhibitory effects of volatile compounds produced by Trichoderma spp. on wood decay fungi

The effect of the production of volatile organic compounds (VOCs) by *Trichoderma* isolates was evaluated with the following techniques as described by DENNIS & WEBSTER (1971). *Trichoderma* isolates were centrally

inoculated by placing 5mm discs on the two different growth media taken from the margin of 7 days old cultures and incubated at 25(±1)°C and 70% relative humidity for 3 weeks. The top of each Petri dish was replaced with the bottom of the MEA plates and than inoculated centrally (5mm discs) with the wood decay fungi. Plates without *Trichoderma* spp. were used as control. Eight replicates were maintained for each treatment. The pairs of each Petri dish were fixed and sealed together with paraffin tape and incubated at 25(±1)°C and 70% relative humidity. Colony diameter of the wood decay fungi was measured after an incubation period of 7 days and the inhibition of mycelial growth was calculated.

Dual culture and interaction tests on wood

Mycoparasitism of all *Trichoderma* isolates against the selected wood decay fungi was assessed in dual culture according to SCHUBERT *et al*. (2008b). The agar disc method was carried out on two different media types, 2% malt extract agar (MEA) and a modified low nutrient medium (LNA) The LNA- medium was selected because of its low C:N ratio which is more representative of the nutritional status of wood (SRINIVASAN *et al*., 1992).

Mycelial discs (5mm) were removed from fresh MEA cultures of each of the 5 wood decay fungi and were placed equidistantly at the margin of Petri dishes (90mm) containing the two media types and then incubated at 25(±1)°C and 70% relative humidity for 3-4 days. Thereafter, discs (5mm) were removed from the margins of actively growing 1-week-old cultures of the *Trichoderma* isolates and placed at opposite sides of the dish, and incubated in the dark at 25(±1)°C and 70% relative humidity for 4 weeks. Petri dishes without antagonistic fungi were used as controls. Six replicates were used for each experiment.

Mycoparasitism was observed in samples removed from the interaction zones according to MOUSSA (2002). Finally the samples were sputter-coated with gold (Cressington Sputter Coater 108auto) and analyzed with a scanning electron microscope (ZEISS DSM 940a).

In addition interaction tests in wood blocks of *Platanus x hispanica* were performed as described by SCHUBERT *et al*. (2008b). For studies of the colonization behaviour, wood blocks were inoculated with two types of conidial suspensions (suspension 1 without additives, suspension 2 with 0.2% glucose and 0.1% urea), placed onto 2-weeks old cultures of the wood decay fungi and incubated in the dark at 25 (±1)°C for 6, 12, 18 weeks. Untreated wood blocks served as controls. Ten replicates were used for each experiment. Analysis of dry weight losses of wood and histological studies of selected wood blocks were performed as described by SCHWARZE & FINK (1998).

Statistical analysis

The results of viable counts are expressed as mean ± SE after log transformation. Mean values among treatments were compared by ANOVA and contrast analysis at 5% ($P<0.05$) and 0.1% ($P<0.001$) level of significance. Correlations were tested using Spearmen's correlation coefficient <rho>. Non parametric variables were measured using the Kruskall-Wallis test at 5% ($P<0.05$). All statistical analyses were performed with SPSS 14 statistical software.

Results

Growth and germination rate under different conditions

The influence of temperature, water activity and growth media on mean growth rate and the germination of *Trichoderma* spp. is provided in Tables 2 and 3. Growth rates of all *Trichoderma* isolates increased with nutritional status of the media (LNA<MEA) as well as with increasing water activity. The latter in particular was a decisive factor. No growth and germination was measured at a_w 0.892 within one week and at a_w 0.955 the growth and germination of all *Trichoderma* isolates was greatly enhanced.

The highest temperature supporting growth was recorded on MEA at 25(±1)°C and on LNA at 30(±1)°C. All *Trichoderma* isolates showed a growth and germination optimum at the highest water activity of a_w 0.998 and at 25(±1)°C. Significant differences between the *Trichoderma* isolates were measured. The highest growth rate was measured for T-126.65 (5.6mm d^{-1}), followed by T-Binab (4.6mm d^{-1}) and T-15603.1 (4.3mm d^{-1}) whereas the highest germination rate was measured for T-15603.1 (37.6%), followed by T-351.93 (37.4%) and T-126.65 (32.8%). The lowest growth and germination rates were observed by T-338.93 ($P<0.001$).

Effect of volatile compounds

The results revealed that after 7 days incubation volatile compounds produced by *Trichoderma* spp. caused a significant inhibition of growth as indicated in Figure 2 ($P<0.05$). No influence of the type of growth media on the mean production and effect of VOCs was detected ($P<0.05$). In addition only three of the *Trichoderma* isolates (T-15603.1 32.8%; T-Binab 28.3%; T352.93 25.7%) were able to significantly inhibit the growth of the wood decay fungi. The weakest effect was recorded for T.338.93 (8.7%). Among varieties of *Trichoderma* spp. concerning the production and effect of VOCs, the wood decay fungi differed significantly in their reaction to the VOCs ($P<0.001$). *I. hispidus* and *G. adspersum* showed a strong sensitivity to the VOCs followed by *P. squamosus.*

TABLE 2. Mean growth rate of the *Trichoderma* spp. under different conditions (mm d^{-1}). ± SE

Temperature	MEA			LNA		
ºC	a_w 0.892	a_w 0.995	a_w 0.998	a_w 0.892	a_w 0.995	a_w 0.998
5	0	0	0	0	0	0
10	0	0	2.9 ± 0.18	0	0	2.7 ± 0.22
15	0	0	8.9 ± 0.21	0	0	7.0 ± 0.16
25	0	6.4 ± 0.15	18.9 ± 0.28	0	7.5 ± 0.16	12.7 ± 0.25
30	0	5.6 ± 0.23	12.9 ± 0.17	0	6.9 ± 0.21	13.1 ± 0.22

TABLE 3. Mean germination rate of *Trichoderma* spp. under different conditions (% d^{-1}). ± SE.

Water activity a_w	Temperature °C				
	5	10	15	25	30
0.892	0	0	0	0	0
0.995	0	0	7.7 ±2.8	52.8 ±13.3	48.5 ± 21.8
0.998	0	14.8 ±6.3	52.7 ±17.1	98.8 ±1.5	98.2 ±2.3

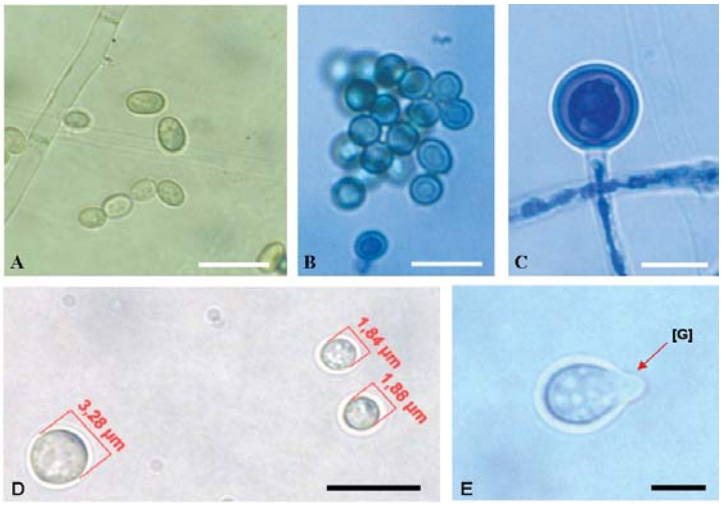

FIGURE 1: **A**: Oval conidia of *T. fasciculatum* (bar, 5µm). **B**: Conidia of *T. atroviride* are spherical (bar, 5µm). **C**: Thick-walled chlamydospore of *T. atroviride* 352.93 (bar, 5µm). **D**: During the process of germination conidia absorbed water and swelled 1,5 fold to their normal dimension (bar, 2µm). E: Germination [arrow] of an inactive occurs via a germ tube resulting in the formation of a hypha (bar, 2µm).

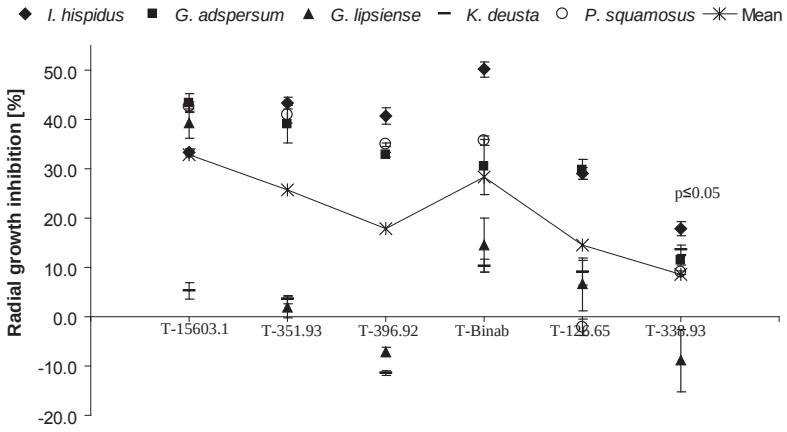

FIGURE 2: Inhibition of radial growth [%] of wood decay fungi by volatile organic compounds (VOC) produced by *Trichoderma* spp.

Evaluation of antagonistic activity on different media

During initial screening of the *Trichoderma* isolates a variety of reactions were recorded as a result of antagonism. Growth of all wood decay fungi, except *P. squamosus*, was inhibited by the *Trichoderma* isolates, although no inhibition zone was observed. Contact between wood decay fungi and *Trichoderma* isolates occurred but the ability to overgrow and to parasitise the mycelia of the wood decay fungi was highly dependent on the antagonistic potential of each *Trichoderma* isolate, their nutritional condition and the resistance of the challenged wood decay fungus to antagonism (Table 4 & 5). The growth medium used had a significant effect on the antagonistic activity ($P<0.05$). The lethal effect of *Trichoderma* spp. was more prevalent on MEA (85.2%) than on the lower nutrient medium (63.7%). The isolates T-126.65 and T-15603.1 showed the strongest antagonistic potential with a statistically similar performance ($P<0.05$). T-338.93, however, had the weakest effect (35%). The highest resistance of wood decay fungi to antagonism of *Trichoderma* spp. was recorded for *P. squamosus. Trichoderma* isolates were able to parasitise the mycelia of *P. squamosus* in only 43% of the cases. *P. squamosus* was not only able to circumvent parasitism but also adapted its hyphal structure, to overgrow the mycelia of the *Trichoderma* isolates (Figure 3A). During parasitism *Trichoderma* spp. showed a target-directed growth towards the mycelia of its hosts and an increased formation of conidiophores, phialides and conidia. Formation of apressoria-like structures enabled the hyphae of *Trichoderma* spp. to attach firmly to the surface of its host mycelia (Figure 3 F&G). Penetration of the mycelia occurred with fine hyphae. The secretion of lytic enzymes and fungicidal substances lead to complete cell wall degradation and efflux of cytoplasm.

Evaluation of antagonistic activity in wood

All wood decay fungi had completely colonized the control wood samples but showed distinctive differences in their potential to decompose the wood. *Kretzschmaria deusta* caused the highest mean dry weight losses (11.7%) followed by the *Ganoderma* species (8.2%), whereas *P. squamosus* (5%) and *I. hispidus* (3.6%) caused the lowest mean weight losses. Only negligible weight losses were recorded from wood samples that were only treated with *Trichoderma* spp. (1.6%).

Analysis of variance showed that the pre-treatment of wood samples with conidial suspensions of *Trichoderma* spp. significantly reduced the mean dry weight losses of all wood decay fungi. When data from treatments with conidial suspension 1 and 2 were compared with the untreated control, significant differences ($P<0.05$) were observed after six weeks

TABLE 4. Classification of the degree of mycoparasitism of different *Trichoderma* spp. on MEA. ± SE.

	MEA					
	T-15603.1	T-351.93	T-396.92	T-Binab	T-126.65	T-338.93
I. hispidus	2.2[a] ± 0.14 [100][b]	2.9 ± 0.98 [100]	2.4 ± 0.65 [83]	2.3 ±0.77 [100]	3.0 ± 0.89 [100]	2.1 ± 0.89 [67]
G. adspersum	3.0 ±0.10 [100]	2.5 ± 0.18 [100]	2.9 ± 0.12 [100]	2.4 ± 0.67 [100]	2.9 ± 0.14 0 [100]	7 ± 0.09 [17]
G. lipsiense	2.3 ±0.11 [83]	2.4 1.23 [100]	1.9 ± 0.21 [67]	1.9 ± 0.23 [83]	2.3 ± 0.54 [100]	0 ± 0.0 [0]
K. deusta	3.0 ±0.36 [100]	2.8 ± 1.31 [100]	2.6 ± 0.33 [100]	2.8 ± 0.42 [100]	2.9 ± 0.56 [100]	1.8 ± 0.36 [67]
P. squamosus	2.2 ±0.56 [83]	1.8 ± 1.05 [67]	2.4 ± 0.66 [83]	1.7 ± 0.11 [83]	2.9 ± 1.45 [100]	0 ± 0.0 [0]

[a] = Following system was used to classify the rate of mycoparasitism: 0 = no overgrowth; 1 = slow overgrowth; 2 = fast overgrowth; 3 = very fast overgrowth and deadlock of the wood decay fungi within 4 weeks.
[b] = Lethal effect as percent was measured by the ability of *Trichoderma* spp. to eliminate the wood decay fungi during the incubation time of 4 weeks.

TABLE 5. Classification of the degree of mycoparasitism of different *Trichoderma* spp. on LNA. ± SE.

	LNA					
	T-15603.1	T-351.93	T-396.92	T-Binab	T-126.65	T-338.93
I. hispidus	1.9[a] ± 0.43 [83][b]	2.4 ± 0.89 [100]	2.2 ± 0.73 [83]	1.9 ±0.09 [100]	1.9 ± 1.32 [100]	1.9 ± 0.10 [67]
G. adspersum	2.3 ± 0.07 [100]	2.2 ± 0.82 [100]	2.4 ± 0.44 [100]	2.1 ± 0.17 [100]	1.8 ± 0.89 [83]	0.8 ± 0.14 [17]
G. lipsiense	1.1 ± 0.33 [17]	1.3 ± 0.07 [33]	0.9 ± 0.69 [17]	0 ± 0.0 [0]	1.8 ± 0.14 [83]	0 ± 0.0 [0]
K. deusta	2.9 ± 0.33 [100]	2.3 ± 0.11 [100]	2.3 ± 0.74 [100]	2.7 ± 0.19 [100]	3.0 ± 1.20 [100]	1.3 ± 0.12 [33]
P. squamosus	0 ± 0.0 [0]	0 ± 0.0 [0]	0.6 ± 0.75 [17]	0 ± 0.0 [0]	2.3 ± 1.01 [83]	0 ± 0.0 [0]

[a] = Following system was used to classify the rate of mycoparasitism: 0 = no overgrowth; 1 = slow overgrowth; 2 = fast overgrowth; 3 = very fast overgrowth and deadlock of the wood decay fungi within 4 weeks.
[b] = Lethal effect as percent was measured by the ability of *Trichoderma* spp. to eliminate the wood decay fungi during the incubation time of 4 weeks.

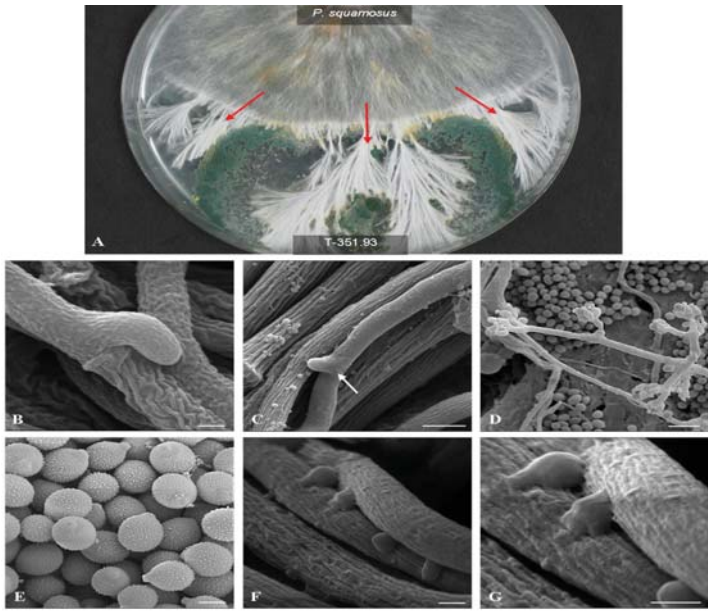

FIGURE 3: **A**: *Polyporus squamosus* was not only able to circumvent parasitism but also formed mycelial strands to overgrow the mycelia of *Trichoderma* isolates **B**: Specific features e.g. clamp connections, typical for basidiomycetes, served to distinguish the mycelia of the wood decay fungi from that of *Trichoderma* (bar, 1µm). **C**: The hyphae of T-396.92 grew target-oriented and branched to increase the contact area with the host mycelium (bar, 5µm). **D + E**: After initial contact an increase in conidiophore (bar, 10µm) and conidial formation (bar, 2µm) by *Trichoderma* spp. was observed. **F + G**: Adhesion of mycelia of wood decay fungi occurred with appressoria-like structures (<1 µm) of *Trichoderma* spp. (bar, 1 µm). The process of parasitism was completed after cell wall degradation and efflux of cytoplasm by secretion of lytic enzymes and fungicidal substances.

of incubation. After 12 and 18 weeks the differences increased and were highly significant ($P<0.001$). The additives used in conidial suspension 2 enhanced significantly the establishment of *Trichoderma* spp. on wood and the protective effect ($P<0.05$). The reduction of wood decay by the *Trichoderma* isolates is illustrated in Table 6 & 7. Contrast analysis of *Trichoderma* isolates revealed significant ($P<0.05$) differences between the species and strains. T-15603.1 induced the greatest reduction in dry weight losses followed by isolates T-351.93 and T-126.65. The isolate T-396.92 and Binab were less effective during the three incubation periods ($P<0.05$). T-338.93 induced the least reduction in weight losses ($P<0.05$).

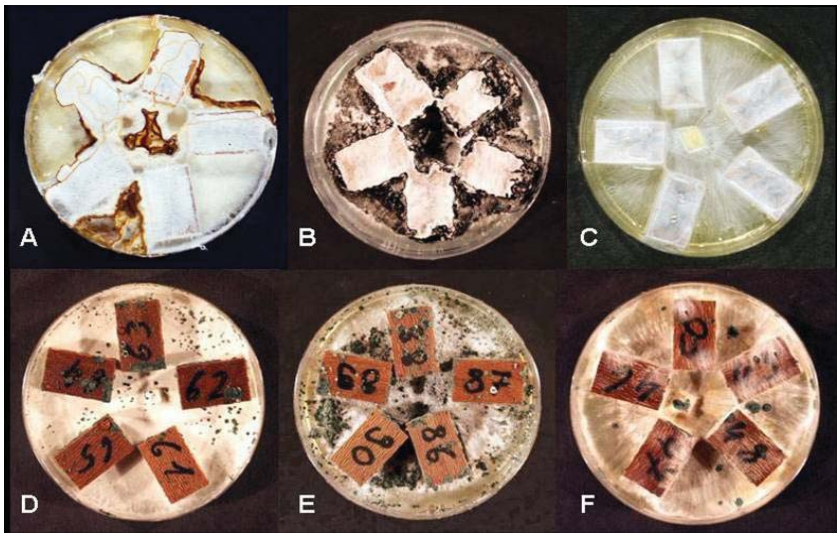

FIGURE 4: **A-C**: Wood samples were completely colonized by *Ganoderma adspersum* (A), *Kretzschmaria deusta* (B) and *Polyporus squamosus* (C) **D**: T-15603.1 inhibited colonization by *Ganoderma adspersum* after 18 weeks of incubation. **E**: Wood pre-treated with T-15603 completely inhibited colonization by *Kretzschmaria deusta*. **F**: Despite pre-treatment with *Trichoderma* spp., *Polyporus squamosus* revealed a high resistance to antagonism and was able to colonize and degrade the wood.

Despite the treatment of wood samples with conidial suspensions of *Trichoderma* spp., *P. squamosus* showed a high resistance to antagonism and caused substantial dry weight losses. All other fungi showed similar performance ($P<0.05$) and sensitivity against *Trichoderma* spp.

Histological analysis supported the results of the macroscopic observations and dry weight loss measurements (Figure 5). High dry weight losses were recorded from control samples by all wood decay fungi, but samples pre-treated with *Trichoderma* spp. did not reveal typical signs of cell wall degradation. *Ganoderma* spp. and *P. squamosus* caused a typical white rot i.e. simultaneous rot and selective delignification. *Inonotus hispidus* showed dual modes of action, i.e. a simultaneous rot and a soft rot, whereas *K. deusta* exclusively caused a soft rot. An alternative degradation pattern was observed for *P. squamosus* on wood pre-treated with *Trichoderma*. Hyphae predominantly grew within intercellular spaces and subsequently degraded the cell wall in close proximity to the hyphae. In wood specimens exclusively inoculated with *Trichoderma* spp. no signs of cell wall degradation were apparent. Hyphae grew predominantly within the parenchyma cells and growth to adjacent cells occurred exclusively via pits.

TABLE 6. Reduction (%) of the wood decay (wood weight loss) by applying conidial suspension 1 of *Trichoderma* spp.

	Conidial Suspension 1														
	Inonotus hispidus			*Ganoderma adspersum*			*Ganoderma lipsiense deusta*			*Kretzschmaria squamosus*			*Polyporus*		
	6 w	12 w	18 w	6 w	12 w	18 w	6 w	12 w	18 w	6 w	12 w	18 w	6 w	12 w	18 w
T-15603.1	62.28*	58.84*	61.58*	59.79*	75.77**	88.40**	85.53**	88.75**	86.24**	75.91**	69.45**	78.76**	55.28*	58.01*	2.55$^{n.s}$
T-351.93	64.07*	60.00*	62.30*	60.82*	76.59**	88.27**	86.81**	88.18**	85.80**	75.77**	71.49**	78.98**	43.09*	56.93*	1.79$^{n.s}$
T-396.92	22.75$^{n.s}$	54.78*	19.75n.s	60.82*	60.33*	74.31**	84.68**	85.04**	73.51**	80.88**	83.10**	78.82**	41.46*	43.29*	–6.64$^{n.s}$
T-Binab	20.96$^{n.s}$	53.33*	17.59n.s	60.82*	60.16*	74.98**	83.83**	85.47**	75.72**	81.17**	83.10**	78.11**	39.84n.s	43.29*	–5.36$^{n.s}$
T-126.65	14.37$^{n.s}$	48.12*	42.73*	57.39*	69.27**	84.29**	68.30*	70.66**	83.66**	74.60**	56.62*	76.19**	55.69*	67.97*	75.35**
T-338.93	10.78$^{n.s}$	34.49$^{n.s}$	49.01*	49.14*	69.76**	81.46**	71.91**	72.79**	80.72**	29.64$^{n.s}$	–11.20$^{n.s}$	30.54$^{n.s}$	28.46$^{n.s}$	–21.65$^{n.s}$	–0.51$^{n.s}$

Significant reduction of the wood decay (wood weight loss) is indicated by * = significant ($P < 0.05$); **=high significant ($P < 0.001$); n.s = not significant ($P \geq 0.05$)

TABLE 7. Reduction (%) of the wood decay (wood weight loss) by applying conidial suspension 2 of *Trichoderma* spp.

	Conidial Suspension 2														
	Inonotus hispidus			*Ganoderma adspersum*			*Ganoderma lipsiense*			*Kretzschmaria deusta*			*Polyporus squamosus*		
	6 w	12 w	18 w	6 w	12 w	18 w	6 w	12 w	18 w	6 w	12 w	18 w	6 w	12 w	18 w
T-15603.1	63.47*	67.25*	73.25*	78.35*	86.83**	91.10**	87.23**	90.17**	86.98**	85.69**	76.78**	82.05**	63.41*	61.04*	16.09$^{n.s}$
T-351.93	64.67*	68.12*	73.43*	78.35*	87.64**	90.96**	86.38**	89.03**	87.12**	85.69**	76.78**	82.38**	64.23*	61.04*	14.81$^{n.s}$
T-396.92	25.15$^{n.s}$	59.71*	52.42*	61.51*	61.46*	81.25**	86.81**	90.17**	79.40**	89.93**	83.30**	78.22**	62.20*	46.75*	17.75$^{n.s}$
T-Binab	25.75$^{n.s}$	59.13*	50.27*	61.86*	61.46*	81.29**	86.91**	89.60**	79.62**	89.34**	83.50**	78.27**	62.60*	47.19*	17.11$^{n.s}$
T-126.65	43.71$^{n.s}$	19.16*	53.62*	67.70*	37.46**	66.83**	81.49*	68.94**	72.22**	74.16**	53.14*	62.83**	70.33*	48.37*	71.21**
T-338.93	52.10$^{n.s}$	59.13$^{n.s}$	69.48*	68.73*	77.89**	88.81**	73.19**	77.64**	83.96**	44.38*	–7.33$^{n.s}$	35.30$^{n.s}$	33.433$^{n.s}$	–6.06$^{n.s}$	5.75$^{n.s}$

Significant reduction of the wood decay (wood weight loss) is indicated by * = significant ($P < 0.05$); ** = high significant ($P < 0.001$); $^{n.s}$ = not significant

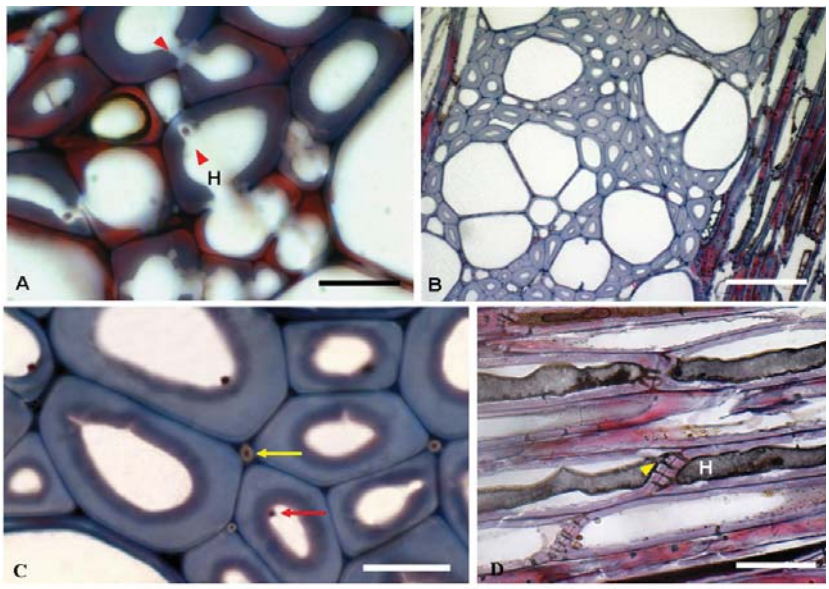

FIGURE 5. **A**: Progressive degradation (simultaneous rot) of the secondary wall by *Ganoderma adspersum* (bar, 5µm). **B**: Wood pre-treated with *Trichoderma* spp. did not show typical signs of cell wall degradation (bar, 50µm). **C**: In pre-treated wood the hyphae of *Polyporus squamosus* predominantly grew within intercellular spaces (yellow arrow) and were rarely observed in the cell lumina (red arrow, bar, 2 µm). **D**: Hyphae of *Trichoderma* spp. were generally located in the parenchyma cells and growth to adjacent cells occurred exclusively via pits (bar, 5µm).

Discussion

Growth and germination under specific conditions

Competitiveness of *Trichoderma* spp. is based on rapid growth and germination i.e. a decisive feature for antagonism (CHET, 1990; CHET *et al*., 1998; HJELJORD & TRONSMO, 1998). Physical as well as chemical factors influence growth and germination, therefore knowledge of the optimal conditions for growth as well as the influence of suboptimal ecological factors on the antagonist is essential for a successful application in field (PAPAVIZAS, 1985; HJELJORD & TRONSMO, 1998; KREDICS *et al*. 2003). In this study, growth of the *Trichoderma* isolates corresponded strongly to the ecological factors tested. All *Trichoderma* isolates showed an optimum growth and germination under an optimized nutritional status, at a mean temperature of 20-25°C and a high water activity of a_w 0.998. At lower temperatures and water activity the growth and germination was significantly reduced to such

a point that at 5°C and a_w 0.892 no growth and germination was recorded after one week. These observations confirm results obtained by KREDICS *et al*. (2000; 2003) and LUPO *et al*. (2002), who classified *Trichoderma* spp. as a mesophilic organism with a low xerotolerance. The prognosis of the behaviour of *Trichoderma* spp. under specific conditions is complicated, however, due to the mutual effect of the environmental parameters (HARMAN, 2006).

Inhibitory effect of volatile organic compounds

Antibiosis in *Trichoderma* was recognized and initially described by WEINDLING (1934) and is defined as the production of secondary metabolites, that have an antimicrobial effect even at low concentrations (HOWELL, 1998). In addition to several other substances (aldehydes, ketones, peptides, etc.), 6-pentyl-α-pyrone (6-PP) is basically responsible for the antifungal effect of the volatile organic compounds (SCARSELLETTI & FAULL, 1994; WHEATLEY *et al*., 1997; COONEY *et al*., 1997a,b; GALINDO *et al*., 2004). SRINIVASAN *et al*. (1992) reported that the composition of the growth media had a significant influence on the production of VOCs and thereby on the levels of inhibition of wood decay fungi by *Trichoderma* spp. However, the results of the present work contrast with these observations, because no significant influence of the growth media type on the mean production and effect of the VOCs could be measured. Significant differences were only detected between different *Trichoderma* isolates. The mean inhibition of 21.4% was low and additionally only 3 of the *Trichoderma* isolates were able to achieve a significant inhibitory effect. This could be an indication for a sub-item of antibiosis concerning the antagonism of *Trichoderma* against wood decay fungi.

Dual culture and interaction tests on wood

In the dual culture tests, hyphal contact between *Trichoderma* spp. and the wood decay fungi was observed for all host/pathogen combinations. However, not all strains of *Trichoderma* were able to overgrow and parasitize the mycelia of wood decay fungi. The antagonistic potential of *Trichoderma* isolates was determined by the nutritional condition of the antagonists and the susceptibility of the wood decay fungi. Previous studies have demonstrated that before mycelia of fungi interact, *Trichoderma* spp. produces low quantities of extracellular exochitinases (KULLNIG *et al*., 2000; BRUNNER *et al*., 2003). The diffusion of these enzymes dissolves cell fragments of host cells. These cell fragments in turn induce the production of further enzymes and trigger a cascade of physiological changes, stimulating rapid and directed growth of *Trichoderma* spp. (ZEILINGER *et al*., 1999). In

the present, work not only directed growth, but also an induced hyphal branching of *Trichoderma* spp. was observed. Previous *in vitro* studies have demonstrated that due to chemotropism hyphae of *Trichoderma harzianum* can grow and branch directly towards the host (CHET, 1987).

In order to increase the antagonistic potential of *Trichoderma* spp. for *in vitro* tests, interaction studies were performed on wood samples. After 18 weeks incubation, treatment with *Trichoderma* spp. failed to completely inhibit decomposition, as measured by dry weight loss. This may partly be explained by the degradation of readily accessible carbohydrates by *Trichoderma* spp. within parenchyma cells and pits (KUBICEK-PRANZ, 1998). A further explanation may be related to the experimental design. Thus wood samples were treated with conidial suspensions of *Trichoderma* and then inoculated with an artificially high inoculum of wood decay fungi. The inoculum potential in turn is crucial for the invasiveness of pathogens (REDFERN & FILIP, 1991). Nevertheless a significant reduction in dry weight losses was induced after pre-treatment of the wood with different conidial suspensions of *Trichoderma* spp. The additives (glucose, urea) stimulated rapid colonization of the wood samples by *Trichoderma* spp. and in their presence the protective effect was increased (HJELJORD *et al*., 2001). In dual culture tests as well as in interaction tests, significant differences between the species and strains of *Trichoderma* spp. were evident. Thus, T-15603.1, T-351.93 and T-126.65 showed a high antagonistic potential. By contrast, the antagonistic potential of T-396.92, the commercial product Binab and especially, T-338.93 was limited.

The different antagonistic activities of the *Trichoderma* strains and the fixed test conditions and the challenged wood decay fungi proved to be decisive factors for the laboratory studies. *In vitro* tests showed that *Polyporus squamosus* is resistant to *Trichoderma* spp. Former studies by SHIELD & ATWELL (1963) and HIGHLEY (1997) demonstrated, without further explanation, that *Trichoderma* spp. have a limited effect on *Polyporus adustus* (Wflld.) Fr. and *Gleophyllum trabeum* (Pers. ex Fr.) Murr. The mechanism that allowed *P. squamosus* to circumvent parasitism in dual culture tests has not been previously described. Formation of hyphal strands by *P. squamosus* was observed after initial contact with hyphae of *Trichoderma* spp. The individual hyphae merged to form compact strands. Thus the surface size was reduced and subsequently the area of hyphae exposed to parasitism. Hyphal strands appeared to be more resistant and enabled *P. squamosus* to readily overgrow the mycelium of *Trichoderma* spp. The resistance of *P. squamosus* hyphae could be due to increased melanin content within the cell wall. DUFFY *et al*. (2003) described melanin as a primary defence system in all organisms and that resistance of pathogenic fungi to microbial lysis is positively correlated with the melanin content in hyphae. During the interaction studies, *P. squamosus* showed specific growth behaviour. Hyphae

of *P. squamosus* were predominantly located within the intercellular spaces escaping mycoparasitism by *Trichoderma* spp. The latter growth pattern has been previously described for *Meripilus giganteus* (Pers. ex Fr.) Karsten (SCHWARZE and FINK, 1998). Thus the basidiomycete was apparently able to circumvent polyphenolic impedances within the reaction zone of beech, *Fagus sylvatica* L. by growing through intercellular spaces.

The limited effect of the commercial product Binab TF WP and the differences in resistance among wood decay fungi in the present study demonstrates the importance of screening *Trichoderma* species for the specific niche where they are envisaged to be applied i.e. increasing target specificity.

The *in vitro* screening of the antagonistic potential used in this work allowed a systematic investigation of several *Trichoderma* isolates including specific ecological factors and a selection of one effective strain. However, positive results obtained from *in vitro* studies are only indicative, as experimental conditions do not take all ecological and endemic factors into account. For this reason field studies are essential to test the selected competitive biocontrol agent under field conditions. The observations and results of field studies with the selected *Trichoderma* strain 15603.1 are reported in SCHUBERT *et al*. (2008a).

Acknowledgments

We would like to thank Mrs. Gack, (Institute for Biology I, University of Freiburg, Germany) and Mr. Kiesel (Institute for Forest zoological Institute; University of Freiburg) for their assistance in scanning electron microscopy (SEM). We would also like to thank Mr. Robert Dietrich and Mr. Karl Merz (Institute for Forest Botany, University of Freiburg) for preparing the wood samples and for technical support. Finally the financial support from the Ev. Studienwerk Villigst e.V. is gratefully acknowledged.

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