

Evaluation of *Trichoderma* spp. as a biocontrol agent against wood decay fungi in urban trees

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Abstract

Laboratory and field tests were performed to establish the potential of *Trichoderma* spp. as a wound treatment for biological control of wood decay fungi in urban trees. A selection of *Trichoderma* species were tested in dual culture and interaction tests in wood against four basidiomycetes *Ganoderma adspersum*, *Ganoderma lipsiense*, *Inonotus hispidus*, *Polyporus squamosus* and one ascomycete *Kretzschmaria deusta*. 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 measured by dry weight loss of wood and qualitatively by histological studies. *Trichoderma atroviride* (T-15603.1) was consistently and highly competitive against most wood decay fungi with the exception of *Polyporus squamosus* which showed resistance towards antagonism in laboratory tests. Field experiments with T-15603.1 were carried out at different locations and hosts. A total of 159 angiosperm trees and 1431 wounds from six different species (*Platanus × hispanica*, *Acer pseudoplatanus*, *Tilia platyphyllos*, *Populus nigra*, *Quercus rubra*, *Robinia pseudoacacia*) were treated with different conidial suspensions. In comparison to control wounds, T-15603.1 induced a highly significant ($P < 0.001$) preventive effect (82.3%) against the selected wood decay fungi. Monitoring results with RAPD-PCR showed that the conidial formulation of 0.1% urea and 0.2% glucose combined with a humidity storing gel as a carrier substance enhanced significantly ($P < 0.001$) the germination rate, conidial viability and therefore the establishment of T-15603.1 in the wood substrate. The results demonstrate that T-15603.1 can be successful following application as biological wound treatment against wood decay fungi on urban sites.

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1. Introduction

The potential of *Trichoderma* species as biocontrol agents of plant pathogens was first recognized in the early 1930s (Weindling, 1932) and subsequently they were applied successfully as biocontrol agents against several plant diseases in commercial agriculture (Howell, 2003). Since the pioneering work of Rishbeth (1961), the interest in the genus *Trichoderma* as competitive antagonists

against important pathogens of forest trees, e.g. *Heterobasidium annosum* (Fr.:Fr.) Bref. and *Armillaria* spp. steadily increased (Holdenrieder, 1984; Nicolotti et al., 1999). Grosclaude et al. (1973) and Corke (1980) successfully applied *Trichoderma viride* Pers. ex S.F. Gray against *Chondrostereum purpureum* (Pers. ex Fr.) Pouzar as a wound treatment method after pruning fruit trees.

Trees on urban sites and along highways and powerlines are pruned for several safety reasons and the incidence of stem decay associated with pruning wounds is high (Gadgil and Bawden, 1982; Wardlaw and Neilsen, 1999; Mohammed et al., 2000). Large volumes of wood decay have an adverse effect on strength and stability and render trees hazardous imposing a high risk to people and property in areas of dense population or high traffic volume.

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As early as 1882, Hartig formulated the objectives of an effective wound treatment for protecting trees against infection and decay after pruning. The concept of using biological wound treatment has become increasingly important in recent years largely due to environmental awareness and dangers to the environment by toxic chemicals. Today a number of commercial products based on *Trichoderma* species such as Binab TF WP, Bio-Innovation, Sweden, exist against a range of pathogens including root and stem decay fungi.

At present, there is limited knowledge regarding the biological control of wood decay fungi which colonize wounds of urban trees. On urban sites air temperature is usually greater than in forests; soil permeability is often low due to compaction causing further stress to the root system of trees. These factors, together with pruning operations, can, alone or in combination, suppress tree health and in the presence of pathogens, trigger disease. Most wood decay fungi that colonize urban trees via pruning wounds rarely occur on forest trees. The unique ecological conditions of urban sites, i.e. the number of pruning wounds inflicted and the low incidence of antagonists due to lack of organic material may promote infection by wound parasites. The objectives of this investigation were to evaluate the potential of different *Trichoderma* species as biocontrol agents, and to identify a competitive species that can be used for the treatment of pruning wounds on urban trees against colonization by wood decay fungi.

2. Materials and methods

2.1. In vitro evaluation of the antagonistic activity

2.1.1. Dual culture tests

The origin of the *Trichoderma* isolates and wood decay fungi are provided in Tables 1 and 2. Several aspects were considered in the selection of the *Trichoderma* isolates. A commercial product was included as well as *T. atroviride* and *T. virens*, which are known from literature to have a high antagonistic potential against several phytopathogenic fungi (Harman and Björkman, 1998; Howell et al., 1997; Kubicek et al., 2001; Hanson and Howell, 2004; Li et al., 2005).

Mycoparasitism of all *Trichoderma* isolates against the selected wood decay fungi was assessed in dual culture according to Highley et al. (1997). The agar disc method was carried out on two different media types, 2% malt extract agar (MEA) and a modified low nutrient medium (LNA) (Huttermann and Volger, 1973 as cited in Freitag, 1989). The LNA-medium was selected because of its low N:C ratio which is more representative of the nutritional status of wood (Srinivasan et al., 1992). Per liter H₂O, it contained: L-asparagine, 0.013 g; KH₂PO₄, 1 g; MgSO₄, 0.3 g; KCL, 0.5 g; FeSO₄, 0.01 g; MnSO₄ 4H₂O, 0.008 g; ZnSO₄ 6H₂O, 0.002 g; CaNO₃ 4H₂O, 0.05 g; CuSO₄, 0.002 g; NH₄NO₃, 0.008 g; D-glucose, 5 g; and agar, 10 g.

Mycelial discs (5 mm) were removed from fresh MEA cultures of each of the five wood decay fungi and were placed equidistantly at the margin of Petri dishes (90 mm) containing the two media types and then incubated at 25(±1) °C and 70% relative humidity for 3–4 days. Thereafter, discs (5 mm) were removed from the margins of actively growing 1-week-old cultures of the *Trichoderma* isolates and placed at the opposite side of the dish, and incubated in the dark at 25(±1) °C and 70% relative humidity for up to 4 weeks. For minimizing unequal mycelia size and therefore differences in the inoculum potential, the growth rates of the fungi were measured before and the data considered in the tests (Holmer and Stenlid, 1993). Petri dishes without antagonistic fungi were used as control. Six replicates were used for each experiment and the experiment was conducted twice.

Petri dishes were examined at regular intervals. The sporulation tufts and pustules of *Trichoderma* fungi were used as an indication for its activity (Naár and Kecskes, 1998). In order to check whether the antagonist was able to overgrow and to parasitize the challenged wood decay fungus, three agar discs (5 mm) were removed from non-sporulating regions and placed on a *Trichoderma*-selective medium TSM (Askew and Laing, 1993). After 7 days of incubation at room temperature, discs were observed for *Trichoderma* colonies. Afterwards, competition values (mycoparasitism rate) were assessed: 0 = no overgrowth; 1 = slow overgrowth; 2 = fast overgrowth; 3 = very fast overgrowth and deadlock of the wood decay fungi). The ability of *Trichoderma* spp. to eliminate the wood decay fungi

Table 1
Origin of *Trichoderma* isolates used in the present study

<i>Trichoderma</i>	Substrata	Isolat-N°
<i>Trichoderma atroviride</i> Karsten	Culture of <i>Armillaria mellea</i> —Germany	15603.1 ^a
<i>Trichoderma atroviride</i> Karsten	Paring of <i>Citrus aurantium</i> —Israel	CBS 351.93 ^b
<i>Trichoderma atroviride</i> Karsten	Forest soil—USA	CBS 396.92 ^b
<i>Trichoderma fasciculatum</i> (<i>strictipile</i>) Bissett ^d	Bark of <i>Betula</i> sp.—The Netherlands	CBS 338.93 ^b
<i>Trichoderma virens</i> Miller, Giddens & Foster	Decayed wood—Germany	CBS 126.65 ^b
BINAB TF WP (<i>T. harzianum</i> / <i>T. polysporum</i>)		IMI 206039/40 ^c

^a Isolate from the Forest Botany, University of Freiburg.

^b Isolates from Centraalbureau voor Schimmelcultures—The Netherlands.

^c BINAB Bio-Innovation AB, Sweden.

^d *T. fasciculatum* synonym *T. strictipile*.

Table 2
Origin of wood decay fungi used in the present study

Pilzart	Substrata	Isolat-Nr.
<i>Polyporus squamosus</i> (Hud.:Fr.) Fr.	ex <i>Tilia cordata</i> Mill.	291101.2 ^a
<i>Ganoderma adspersum</i> (S. Schulz.) Donk	ex <i>Fagus sylvatica</i> L.	086699.2 ^a
<i>Ganoderma lipsiense</i> (Batsch) Atk.	ex <i>Fagus sylvatica</i> L.	250593.1 ^a
<i>Inonotus hispidus</i> (Bull.:Fr.) Karsten	ex <i>Fraxinus excelsior</i> L.	200792.1 ^a
<i>Kretzschmaria deusta</i> (Hoffm.) P.M.D. Martin	ex <i>Acer pseudoplatanus</i> L.	271098.1 ^a

^a Isolate from the Forest Botany, University of Freiburg.

(lethal effect) during the time of incubation was evaluated by aseptically transferring 5 mm discs from test plates to a MEA medium with 2 ml thiabendazole (2-(4'-thiazolyl)-benzimidazole), Merck, Darmstadt, Germany (0.46 mg dissolved in lactic acid). T-MEA suppresses growth of *Trichoderma* spp. but allows growth of wood decay fungi (Sieber, 1995). The lethal effect of *Trichoderma* spp. was expressed as percentage of the eliminated wood decay fungi.

Mycoparasitism was observed in samples removed from the interaction zones according to Moussa (2002). Samples were fixed in 4% glutaraldehyd, 0.1 M phosphate buffer, pH value 7.2–7.4, and stored at 4 °C for 12 h. Dehydration and desiccation was undertaken with an increasing concentration of isopropanol (50%, 60%, 70%, 80%, 90% and 2P × 100%) for 10 min. Subsequent to desiccation, the samples were dried by critical point drying (Bal-Tec CPD 030). Finally the samples were sputted with gold (Cressington Sputter Coater 108auto) and analyzed with a scanning electron microscope (Zeiss DSM 940a).

2.1.2. Interaction tests on the wood substrate

Trichoderma isolates were tested on London plane (*Platanus × hispanica* Münchh.) wood blocks (10 × 15 × 25 mm) according to the European Standard EN 113 (1996) with slight modifications. For determination of dry wood weight losses three wood blocks were dried for 48 h at 103(±1) °C. Wood blocks were stored in a vacuum chamber until they reached a moisture content of 50–70% and were then autoclaved twice for 20 min at 121(±1) °C. Two types of conidial suspensions of *Trichoderma* spp. were used for wood inoculation. Suspension 1 contained approx 10⁵ CFU/ml and suspension 2 contained 0.2% D-glucose (Merck) and 0.1% urea (Riedel–DeHaën) in addition to 10⁵ CFU/ml. Wood blocks were inoculated with the conidial suspensions and then placed onto 2-week-old cultures of the wood decay fungi (five wood blocks per Petri dish containing MEA). Untreated wood blocks served as controls. Wood blocks were inoculated with conidial suspensions of the *Trichoderma* isolates and incubated in the dark at 25(±1) °C for 6, 12 and 18 weeks to study colonization behavior. Ten replicates were used for each experiment and the experiment was conducted twice. Analysis of dry weight losses of wood and histological studies of selected wood blocks were performed as described by Schwarze and Fink (1998).

2.2. Field studies

Field experiments were undertaken on urban sites in Freiburg–Lehen, Baden–Württemberg (278 m above sea level, 10 °C; precipitation approx 100 cm per annum) and in Ludwigshafen, Rheinland-Pfalz (96 m above sea level, 9.2 °C; precipitation approx 500 mm per annum), Germany. A total of 159 trees were pruned (54 *Platanus × hispanica* Münchh., 40 *Acer pseudoplatanus* L., 24 *Tilia platyphyllos* Scop., 16 *Populus nigra* L., 16 *Quercus rubra* L., 9 *Robinia pseudoacacia* L.). A total of 1431 wounds (nine per tree) had a mean diameter of 6.4 cm (range 1–45 cm) and were treated in July and August 2003 with three different treatments consisting of conidial suspensions of the antagonist T-15603.1:

- (1) Suspension (CFU: 10⁵/ml).
- (2) Suspension (CFU: 10⁵/ml + 0.2% D-glucose + 0.1% urea).
- (3) Suspension (CFU: 10⁵/ml + 0.2% D-glucose + 0.1% urea + 0.4% sodium polyacrylate, a component of the product Luquasorb 1030, BASF AG Ludwigshafen, Germany).

Treatments 1 and 2 were sprayed on the wound surface; the third treatment was applied with a conventional brush (approx 2–3 ml). Untreated wounds served as control.

For evaluation of the protective effect of the *Trichoderma*-strains, artificial inoculation tests were performed on selected trees. Tree wounds were treated as described above and 3 weeks after treatment they were inoculated with one of the three basidiomycetes: *I. hispidus*, *G. adspersum* and *P. squamosus*. Mycelia of the fungi were harvested, filtered and dissolved in sterile water (2 g/ml). The inoculations were performed by applying high inocula (high amount of infective material) of the wood decay fungi (approx 3–4 ml of mycelia suspension). The determination of the percentage of infected wounds (infection rate) by these fungi was based on inoculated wounds from which the respective fungus could be re-isolated after 30 months (Gadgil and Bawden, 1982). Sections of the treated wounds were extracted and bisected radially. Re-isolations were performed by extracting wood samples (5 × 5 × 5 mm) from the inoculated wounds under sterile conditions. The samples were placed on T-MEA and the identification of the re-isolated wood decay fungi was undertaken with

conventional methods based on macro- and micro-morphological features (Stalpers, 1978).

2.2.1. Re-isolation and analysis

Establishment and the persistence of the *Trichoderma*-strain in the wood substrate were monitored with standardized re-isolations after 2, 8, 12, 18, 24 and 30 months. Wood samples (20 × 10 × 5 mm) were extracted from the center and periphery of the wounds with a sterile chisel. In the laboratory, the surface of the samples were sterilized with hydrogen peroxide, divided into three parts and placed onto MEA, TSM and T-MEA.

In addition, after 30 months sections of the treated wounds were extracted, bisected radially and re-isolations were performed as described above. Callus growth, discoloration in areas of the wounds and the effectiveness of the antagonist were recorded and measured (Liese et al., 1988; Metzler, 1997). Data on the site, orientation, climate, ratio of sapwood to heartwood and wound dimension were also recorded periodically.

A statistical interpretation and characterization of the isolated fungal populations such as diversity and succession were analyzed for diversity indices (Maria and Sridhar, 2002).

$$\text{Shannon Index } H' : H' = - \sum_{i=1}^S pi \times \log(pi), \quad pi = \frac{ni}{N}$$

S = number of different species or groups; N = total numbers of individuals; ni = number of individuals belonging the i th species or group; pi = proportion of total sample belonging to i th species.

The Shannon equitability or evenness index (E) can be defined as the ratio between the observed species diversity (H') and the maximum species diversity (H_{\max}) and was expressed by: $E = \frac{H'}{H_{\max}} = \frac{H'}{\log(S)}$. E = equitability (range 0–1, max. value when individuals evenly distributed among the species or groups); H = observed species diversity; H_{\max} = species diversity under conditions of maximal evenness.

2.2.2. Morphological and molecular identification

Identification of the obtained isolates was undertaken both with conventional methods based on macro- and micro-morphological features and with RAPD-PCR as described by Castle et al. (1998).

Morphological observations were made from cultures grown on 2% MEA at 25(±1) °C under ambient laboratory conditions and diffuse daylight. The microscopic characteristics were observed for the more complex conidiophores developing from characteristic tufted or pustuled areas with conidiophores. Identity was established using several diagnostic keys (Rifai, 1969; Bissett, 1984, 1991a,b,c, 1992; Gams and Bissett, 1998).

2.3. Random amplified polymorphic DNA (RAPD)

For DNA isolation fungal cultures were grown at room temperature on 2% MEA. Mycelia were harvested by filtra-

tion through a piece of filter paper and washed with distilled water. The samples were frozen immediately in liquid nitrogen and lyophilized. DNA extractions were carried out with a PhytoPure DNA extraction kit (Amersham Life Science, Buckinghamshire, UK) according to the manufacturer's instructions. The DNA concentration was determined by measuring the absorbance at 260 nm (Beckmann DU 7500i, Munich).

RAPD characters were developed with primer 1 (5'-CA CGGCGAGT-3') and primer 2 (5'-CTGTCCAGCA-3') (Carl Roth GmbH). The reaction volume of 50 µl contained 26.5 µl distilled water, 5 µl Mg-reaction buffer, 5 µl 25 mM MgCl₂, 5 µl Primer 1 and 2, 1 µl 10 mM dNTP mix, 0.5 µl *Taq* DNA polymerase, 2 µl DNA (0.5 ng/µl). PCR amplification was performed in a Eppendorf Mastercycler Gradient (Eppendorf AG, Hamburg) programmed for 1 cycle of initial denaturation for 3 min at 94 °C, 7 min at 74 °C, followed by 39 cycles of 1 min at 94 °C (denaturation), 1 min at 37 °C (low stringency annealing), 2 min at 72 °C (elongation) with a final extension step for 10 min at 72 °C. The DNA samples were separated for analysis by electrophoresis on 1–2% agarose gels (1 × Tris–borate EDTA buffer). The fragments were visualized by staining with 1 µl of ethidium bromide (10 mg/ml) per ml agarose gel and UV illumination.

2.4. Statistical analysis

The results of viable counts are expressed as means ± SE after log transformation. GLM (General Linear Model) repeated measures analysis of variance (ANOVA) was used to assess the impact of different treatments (conidial suspensions); means were separated using contrast statements at significance level of $P < 0.05$. Data expressed as percentages were arcsine-transformed prior to analysis and back-transformed to numerical values. Non-parametric variables such as the assessed competition values were analyzed by using the Kruskal–Wallis test at $P < 0.05$. Correlations were tested using Spearman's rho ($\langle \rho \rangle$). The statistical package used for all analyses was Systat 10 (Systat, 2000).

3. Results

3.1. In vitro evaluation of antagonistic activity on artificial 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 in all cases but analysis of variance showed that the growth medium used ($P < 0.05$) as well as the *Trichoderma* isolates ($P < 0.001$) and hosts ($P < 0.001$) had a significant effect on the outcome of antagonism. The antagonistic activities

of *Trichoderma* spp. were more prevalent on MEA (mean competition value 2.2; and lethal effect 82.7%) than on the lower nutrient medium (1.5; 60.5%). The rates of mycoparasitism by *Trichoderma* isolates on MEA and LNA are indicated in Tables 3 and 4. Analyses by the Kruskal–Wallis test revealed that the isolate T-126.65 had the highest antagonistic potential with a competition value of 2.48 and the ability to eliminate (lethal effect) the wood decay fungi in 97% of the cases followed by the isolates T-15603.1 (2.1; 87.5%) and T-351.93 (2.06; 82.6%) with a statistically similar performance ($P < 0.05$). T-338.93, however, showed the lowest effect (0.86; 35%). The highest resistance of wood decay fungus to *Trichoderma* spp. was recorded from *P. squamosus*. *Trichoderma* isolates were able to parasitize and eliminate the mycelia of *P. squamosus* in only 43% (69.3% on MEA; 16.7% on LNA) of the cases. *P. squamosus* not only circumvented parasitism but also adapted its hyphal structure to overgrow the mycelia of the *Trichoderma* isolates (Fig. 1).

During parasitism, hyphae of *Trichoderma* spp. showed a target-directed growth towards the mycelia of the hosts and induced hyphal branching. Formation of apressorium like structures enabled the hyphae of *Trichoderma* spp. to firmly attach to the surface of its host mycelia (Fig. 2). 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.

3.2. In vitro evaluation of antagonistic activity in wood

After 6 weeks incubation, the wood decay fungi had completely colonized the control wood samples and dry

weight losses increased with time. Wood decay fungi 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%), *P. squamosus* (5%) whereas *I. hispidus* (3.6%) caused the lowest mean weight losses. Only negligible weight losses (1.6%) were recorded from wood samples, which were merely treated with *Trichoderma* spp. Analysis of variance showed that a treatment of wood samples with conidial suspensions of *Trichoderma* spp. significantly reduced the mean dry weight losses by all wood decay fungi. Data shown in Table 5 shows the contrast analysis of different conidial suspensions tested. When data from treatment with conidial suspensions 1 and 2 were compared with the untreated control, significant differences ($P < 0.05$) were observed after 6 weeks incubation and after 12 and 18 weeks the differences increased and were highly significant ($P < 0.001$). Comparisons between conidial suspension 1 (treatment 1) without additives and conidial suspension 2 (treatment 2) with 0.2% D-glucose and 0.1% urea were not significant after 6 weeks, but after 12 and 18 weeks of incubation ($P < 0.05$). The additives appeared to enhance the growth of *Trichoderma* spp. and to facilitate the colonization of the wood specimens. The reduction of wood weight loss by *Trichoderma* isolates is illustrated in Table 6. Contrast analysis of *Trichoderma* isolates revealed significant ($P < 0.05$) differences between the species and strains. After 6, 12 and 18 weeks T-15603.1 induced the highest reduction in dry weight losses followed by isolates T-351.93 and T-126.65 with statistically a similar performance ($P < 0.05$). The isolate T-396.92 and Binab also showed a statistically similar performance but were less

Table 3
Classification of the mycoparasitism of *Trichoderma* spp. on MEA \pm SE

	T-15603.1	T-351.93	T-396.92	T-338.93	T-126.65	BINAB
MEA						
<i>I. hispidus</i>	2.2 ^a \pm 0.14 [100] ^b	2.9 \pm 0.98 [100]	2.4 \pm 0.65 [83]	2.1 \pm 0.89 [67]	3.0 \pm 0.89 [100]	2.3 \pm 0.77 [100]
<i>G. adspersum</i>	3.0 \pm 0.10 [100]	2.5 \pm 0.18 [100]	2.9 \pm 0.12 [100]	0.7 \pm 0.09 [17]	2.9 \pm 0.14 [100]	2.4 \pm 0.67 [100]
<i>G. lipsiense</i>	2.3 \pm 0.11 [83]	2.4 \pm 1.23 [100]	1.9 \pm 0.21 [67]	0 \pm 0.0 [0]	2.3 \pm 0.54 [100]	1.9 \pm 0.23 [83]
<i>K. deusta</i>	3.0 \pm 0.36 [100]	2.8 \pm 1.31 [100]	2.6 \pm 0.33 [100]	1.8 \pm 0.36 [67]	2.9 \pm 0.56 [100]	2.8 \pm 0.42 [100]
<i>P. squamosus</i>	2.2 \pm 0.56 [83]	1.8 \pm 1.05 [67]	2.4 \pm 0.66 [83]	0 \pm 0.0 [0]	2.9 \pm 1.45 [100]	1.7 \pm 0.11 [83]

^a Following system was used to rate 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 4
Classification of mycoparasitism of *Trichoderma* spp. on LNA \pm SE

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

^a Following system was used to rate 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.

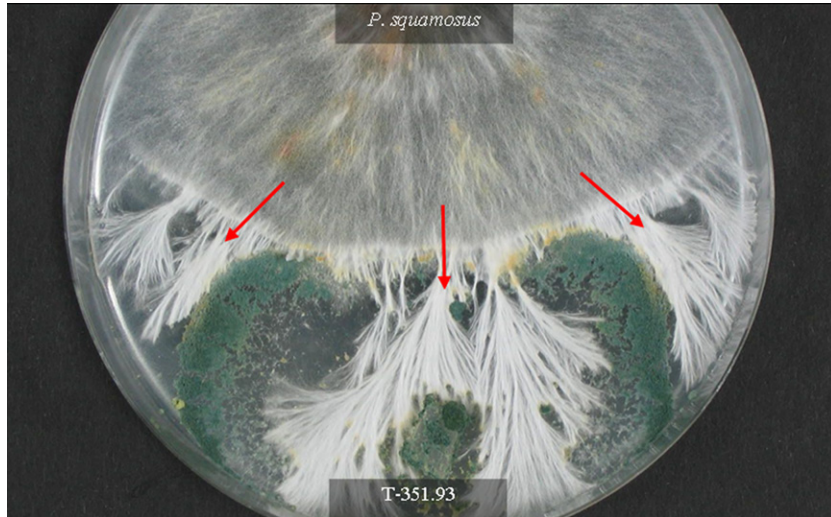


Fig. 1. In dual culture tests *Polyporus squamosus* overgrew the mycelia of *Trichoderma* spp. by adapting its hyphal structure to compact strands (red arrows).

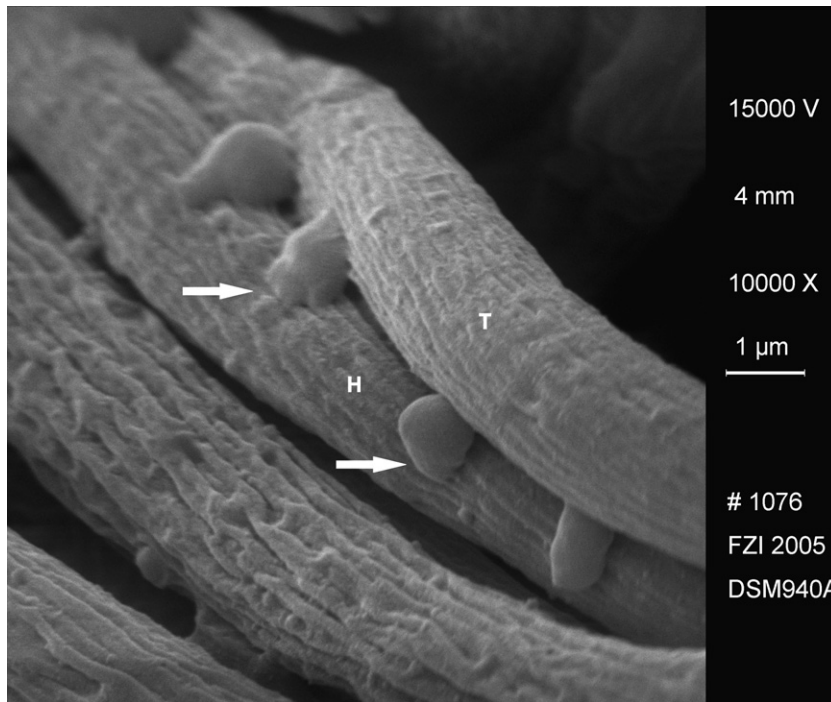


Fig. 2. Hyphae of T-15603.1 [T] grew target-oriented and formed appressoria-like structures (arrows) that enabled hyphae [T] to adhere to the host [H].

Table 5
Contrast analysis of different conidial suspensions of *Trichoderma* spp. for the control of wood decay fungi

Methods of conidial suspensions	Time [weeks]		
	6	12	18
Suspension 1 ^a vs. control	0.043	0.021	0.001
Suspension 2 ^b vs. control	0.345	0.009	0.000
Suspension 2 ^b vs. suspension 1 ^a	0.073	0.042	0.034

F-test of significance $P < 0.05$, P value is denoted, not significant at $P \geq 0.05$.

^a Conidial suspension (CFU 10^5 ml⁻¹) without additives.

^b Conidial suspension (CFU 10^5 ml⁻¹) with 0.1% urea; 0.2% glucose.

effective ($P < 0.05$) during the three incubation periods, whereas T-338.93 induced the lowest reduction ($P < 0.05$).

With time wood decay fungi revealed differences in their sensitivity to *Trichoderma* treatment. Contrast analysis among the wood decay fungi revealed a significant difference ($P < 0.001$) after 6, 12 and 18 weeks incubation for *P. squamosus* and the other wood decay fungi. Despite the treatment of wood samples with conidial suspensions of *Trichoderma* spp., *P. squamosus* showed high resistance and caused substantial dry weight losses (62.8%). All other fungi showed statistical similar performance ($P < 0.05$) and sensitivity against *Trichoderma* spp.

Table 6

Reduction of wood weight loss (%) by wood decay fungi after applying different conidial suspension of *Trichoderma* spp. \pm SE

	Conidial suspension 1 ^a			Conidial suspension 2 ^b			Mean
	6 weeks	12 weeks	18 weeks	6 weeks	12 weeks	18 weeks	
T-15603.1	67.8 \pm 4.27	70.2 \pm 4.17	69.2 \pm 4.03	75.6 \pm 4.22	76.4 \pm 4.22	82 \pm 4.17	73.5
T-351.93	66.9 \pm 4.07	68.3 \pm 4.03	63 \pm 3.94	72.4 \pm 4.22	70.2 \pm 4.17	72.1 \pm 4.17	68.8
T-396.92	55.8 \pm 3.98	60.9 \pm 4.03	59.2 \pm 4.17	63.4 \pm 4.22	66.7 \pm 4.22	64.2 \pm 4.27	61.7
T-338.93	38 \pm 4.03	28.8 \pm 4.07	48.3 \pm 3.94	54.3 \pm 4.07	42.7 \pm 4.03	56.7 \pm 4.03	44.8
T-126.65	54.1 \pm 3.94	62.5 \pm 3.98	72.5 \pm 3.90	67.5 \pm 4.07	70.1 \pm 4.12	81.2 \pm 4.07	68.0
BINAB	57.3 \pm 4.27	65.1 \pm 4.12	48.2 \pm 4.07	65.1 \pm 4.17	68.2 \pm 4.03	61.4 \pm 4.07	60.9

^a Conidial suspension (CFU 10⁵ ml⁻¹) without additives.^b Conidial suspension (CFU 10⁵ ml⁻¹) with 0.1% urea; 0.2% glucose.

The histological analysis supports the results of the macroscopic observations and dry weight loss measurements. High dry weight losses were recorded from control samples by all wood decay fungi, but samples 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* caused 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 of *P. squamosus* predominantly grew within intercellular spaces and subsequently degraded the cell wall in close proximity to the hyphae (Fig. 3). In wood specimens exclusively inoculated with *Trichoderma* spp. no signs of cell wall degradation were apparent. Hyphae of *Trichoderma* spp. grew predominantly within the parenchyma cells and growth to adjacent cells occurred exclusively via pits.

3.3. Field trails

Monitoring results revealed re-isolation of the *Trichoderma* strain T-15603.1 from treated wounds 30 months after application (Fig. 4). The establishment and the successful colonization of the wound surface by T-15603.1

were highly dependent on the medium in which the conidia were suspended (Table 7). Table 8 shows the contrast analysis of different conidial suspensions tested. The significantly highest mean re-isolation rate of 74.8% was obtained after application with conidial suspension 3 ($P < 0.05$). Wounds were much weaker colonized after treatment with suspensions 1 (32.5%) and 2 (29.1%), that showed similar statistical performance. Monitoring results of re-isolation rates from wounds treated with suspension 3 were significantly higher than from control wounds ($P < 0.001$). After 12 months no differences between treatment of wounds with suspension 2 and control wounds was determined. After 30 months no differences were observed between the re-isolation rates of suspension 1 and the controls. Correlation analysis according to Spearman's rho showed a positive relationship between precipitation and re-isolation rate ($\langle \rho \rangle = 0.829$, $P < 0.05$) and in a negative correlation between wound dimension and re-isolation rate ($\langle \rho \rangle = 0.714$, $P < 0.05$). In comparison to re-isolations from sapwood wounds (69.7%), an increase in wound size and proportion of heartwood resulted in a lower re-isolation rate (30.3%). No statistically significant correlation between re-isolation rate and temperature was detected ($\langle \rho \rangle = 0.371$, $P < 0.05$) neither between re-isolation rate or tree species ($\langle \rho \rangle = 0.276$, $P < 0.05$).

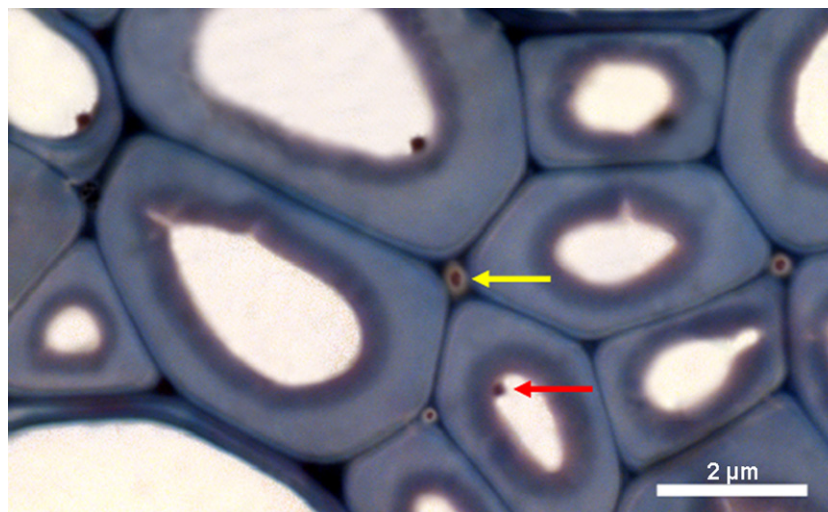


Fig. 3. Transverse section of London plane wood incubated with T-15603.1 and *Polyporus squamosus* (magnification 400 \times). Note hyphal growth of *Polyporus squamosus* in the cell lumina (red arrow) and also within intercellular spaces (yellow arrow).

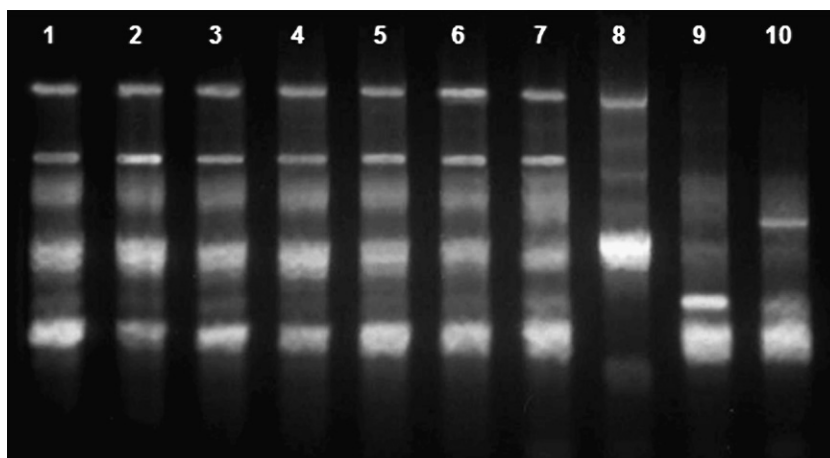


Fig. 4. RAPD-PCR: (1) = The applied strain T-15603.1 (reference). (2–7) = *Trichoderma* spp. isolated from the treated tree wounds after 30 months. (8–10) = *Trichoderma* spp. isolated from untreated tree wounds.

Table 7

Re-isolation rate of T-15603.1 (%) from pruning wounds in relation to the different conidial suspensions applied

	2 months	8 months	12 months	18 months	24 months	30 months	Mean
Control ¹	3.3	4.5	4.8	4.7	5	4.8	4.5
Suspension 1 ^a	31.5	52.5	30	27	27.8	26.4	32.5
Suspension 2 ^b	50	32.5	25	22.5	23	21.4	29.1
Suspension 3 ^c	81	82.5	72.5	70	72	71	74.8

^a Conidial suspension (CFU 10⁵ ml⁻¹) without additives.

^b Conidial suspension (CFU 10⁵ ml⁻¹) with 0.1% urea; 0.2% glucose.

^c Conidial suspension (CFU 10⁵ ml⁻¹) with 0.1% urea; 0.2% glucose and 0.4% sodium polyacrylate.

Table 8

Contrast analysis of re-isolation rate of T-15603.1 from pruning wounds in relation to the different conidial suspensions applied

Methods of conidial suspensions	Time [months]					
	2	8	12	18	24	30
Suspension 1 ^a vs. control	0.038	0.001	0.042	0.045	0.048	0.056
Suspension 2 ^b vs. control	0.001	0.046	0.052	0.058	0.078	0.167
Suspension 3 ^c vs. control	0.001	0.001	0.000	0.000	0.000	0.000
Suspension 2 ^b vs. suspension 1 ^a	0.055	0.061	0.089	0.092	0.104	0.096
Suspension 3 ^c vs. suspension 1 ^a	0.000	0.019	0.001	0.001	0.001	0.001
Suspension 3 ^c vs. suspension 2 ^b	0.014	0.000	0.001	0.001	0.000	0.000

F-test of significance $P < 0.05$, P value is denoted, not significant at $P \geq 0.05$.

^a Conidial suspension (CFU 10⁵ ml⁻¹) without additives.

^b Conidial suspension (CFU 10⁵ ml⁻¹) with 0.1% urea; 0.2% glucose.

^c Conidial suspension (CFU 10⁵ ml⁻¹) with 0.1% urea; 0.2% glucose and 0.4% sodium polyacrylate.

In addition to bacteria a range of fungal genera and species were isolated from the untreated wounds (Table 9). The application of T-15603.1 reduced the microbial diversity of colonized wounds (Fig. 5). The mean indices of the untreated wounds (control) were $H' = 1.547$ and $E = 0.846$ higher than the indices of the treated wounds ($H' = 0.829$ and $E = 0.414$). Wounds treated with suspension 3 showed the lowest diversity indices (suspension 1 $H' = 0.811$, $E = 0.468$; suspension 2 $H' = 1.024$, $E = 0.534$; suspension 3 $H' = 0.651$, $E = 0.242$). Only 25 genera of fungi but no basidiomycetes were isolated from the treated wound which is equivalent to a reduction in microbial diversity of 28.6%.

The evaluation of the artificial inoculation tests showed high biocontrol efficacy of T-15603.1 against *I. hispidus*, *G. adspersum* and *P. squamosus* on pruning wounds (Fig. 6). Control wounds that were inoculated with wood decay fungi but not treated with *Trichoderma*, showed a mean infection rate of 78.7%. *G. adspersum* caused the highest infection rate (81%) followed by *P. squamosus* (79%) and *I. hispidus* (76%). All fungi showed a similar statistical performance ($P < 0.05$). Analysis of variance showed that the treatment of pruning wounds with conidial suspension of T-15603.1 reduced the colonization significantly ($P < 0.001$) from 78.7% to 13.9%; which corresponds to a mean wound treatment efficiency of 82.3%. T-15603.1

Table 9
Fungal genera and species isolated from untreated pruning wounds

Deuteromycetes		Ascomycetes	Zygomycetes	Basidiomycetes	Myxomycetes
<i>Acremonium</i> sp.	<i>Pestalotia</i> sp.	<i>Aleuria</i> sp.	<i>Absidia</i> sp.	<i>Flammulina velutipes</i>	<i>Physarum</i> sp.
<i>Alternaria</i> sp.	<i>Penicillium</i> sp.	<i>Apiognomonia</i> sp.	<i>Mortierella</i> sp.	<i>Stereum hirsutum</i>	
<i>Aureobasidium</i> sp.	<i>Phialophora</i> sp.	<i>Ascocoryne</i> sp.	<i>Rhizopus</i> sp.	<i>Trametes versicolor</i>	
<i>Cladosporium</i> sp.	<i>Phoma</i> sp.	<i>Chaetomium</i> sp.			
<i>Curvularia</i> sp.	<i>Nodulisporium</i> sp.	<i>Daldinia</i> sp.			
<i>Epicoccum</i> sp.	<i>Stemphylium</i> sp.	<i>Erysipha</i> sp.			
<i>Exophiala</i> sp.		<i>Hypocrea</i> sp.			
<i>Fusarium</i> sp.		<i>Nectria</i> sp.			
<i>Gliocladium</i> sp.		<i>Peziza</i> sp.			
<i>Monocillium</i> sp.		<i>Verticillium</i> sp.			
<i>Paecilomyces</i> sp.		<i>Xylaria</i> sp.			

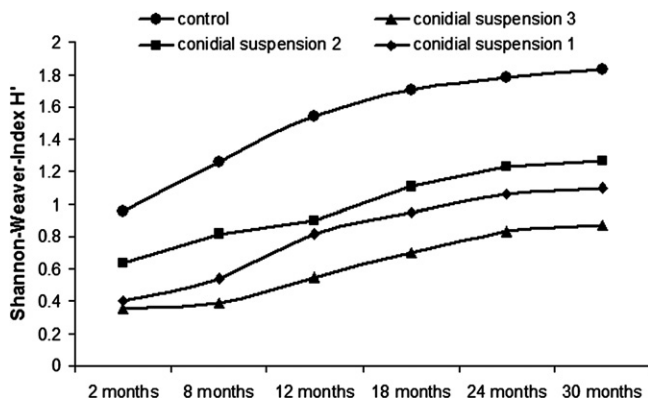


Fig. 5. Diversity index H' calculated from wounds treated with different conidial suspensions of T-15603.1 (conidial suspension 1 —◆—; conidial suspension 2 —■—; conidial suspension 3 —▲—) and untreated control wounds (—●—) after 2, 8, 12, 18, 24 and 30 months.

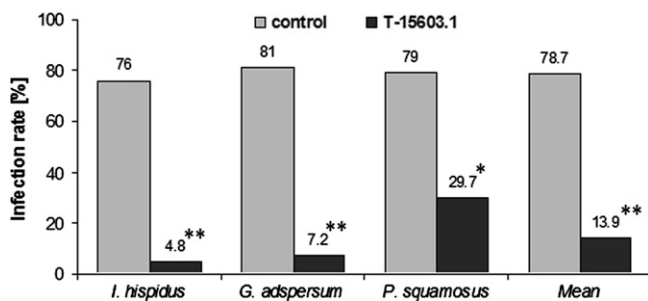


Fig. 6. Reduction of the infection rate of wood decay fungi on pruning wounds after treatment with T-15603.1. Significant differences (F -test) between control and treatment are indicated by * $P < 0.05$; ** $P < 0.001$.

showed the highest efficiency (93.7%; $P < 0.001$) against *I. hispidus*. A similar reduction in infection rate (91.1%; $P < 0.001$) was also determined for *G. adspersum*, whereas efficacy against *P. squamosus* (62.4%; $P < 0.05$) was limited.

4. Discussion

Isolates of *Trichoderma* spp. have been demonstrated to be antagonistic toward a number of fungi (Howell, 2003). In the present work, a hierarchical set of assays including

dual culture tests (a) on different media and interaction tests (b) on wood were used to identify one effective *Trichoderma* isolate against wood decay fungi. Identification and differentiation (c) of the isolate were performed using both classical and molecular methods and field experiments (d) were carried out to test different conidial suspensions of the selected isolate under natural conditions.

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 interacted, *Trichoderma* spp. produced low quantities of extracellular exochitinases (Kullnig et al., 2000; Brunner et al., 2003). The diffusion of these enzymes dissolved cell fragments of host cells. These cell fragments in turn induced the production of additional enzymes and triggered 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. Previously, *in vitro* studies have shown that due to chemotropism *Trichoderma harzianum* hyphae grew and branched directly towards their host (Chet, 1987).

In order to increase the antagonistic potential of *Trichoderma* spp. in the *in vitro* tests, interaction studies were performed on wood samples according to European Standard EN 113 (1996). After 18 weeks incubation, treatment with *Trichoderma* spp. failed to inhibit decomposition completely, as measured by dry weight loss. This may be explained partly 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 and then inoculated with an artificially high amount of inoculum of wood decay fungi. The inoculum potential in turn was crucial for the invasiveness of the pathogens (Redfern and Filip, 1991). Nevertheless a significant reduction in dry

weight losses was obtained using different conidial suspensions of *Trichoderma* spp. The addition of glucose and urea caused faster 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 (C/N-source, Fe-source, P-availability, pH value, temperature, water regime) 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. Earlier studies by Shields and Atwell (1963) and Highley (1997) demonstrated, without further clarification, that *Trichoderma* spp. had a limited effect on *Polyporus adustus* (Wfild.) 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. A modification in hyphal structure of *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 cell wall melanin content. Duffy (2003) suggested that melanin is 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 particular growth behavior. 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 the wood decay fungi demonstrates the importance of screening *Trichoderma* species for the specific niche where they shall be applied subsequently increasing target specificity.

The field screening step was necessary to demonstrate the potential of the selected *T. atroviride* isolate 15603.1 for biological control of wood decay fungi under field conditions. Optimal establishment of the antagonist in the wood substrate before a pathogen arrives appears to be a good strategy for a successful wound protection treatment. However establishment is dependent on the adhesion and

the viability of the conidia of the biocontrol agent and this is more difficult in the natural environment than in conditions found in the laboratory. One way to improve performance of biocontrol agents is by formulating conidia to enhance the adhesion and the viability. In this study three different conidial suspensions of T-15603.1 were formulated and tested, which significantly influenced the establishment ($P < 0.001$). The concentration of all suspensions was adjusted to 10^5 CFU/ml. Hjeljord et al. (2001) observed that higher concentrations of conidia resulted in a reduction in conidial germination. Conidial suspension 2 (Treatment 2) was amended with urea and glucose. Hjeljord et al. (2001) showed that the addition of nutrients (C and N sources) resulted in an increase in germination rate and conidia viability and that there was a statistically significant relationship between increased germination rate, viability and biocontrol efficacy of *Trichoderma* spp. However the authors also mentioned that conidia that were enriched with nutrients showed an increased water requirement resulting in reduced germination due to lowered water potential. A comparison with climate data showed a significant correlation between re-isolation rate and precipitation on different sites. This may explain why no significant effect ($P < 0.05$) could be observed between the amended suspension 2 and the non-amended suspension 1. The viability of the enriched conidia was limited due to increased water requirement and by desiccation of the wood substrate, therefore the establishment, as indicated by re-isolation rate of T-15603.1, was reduced. Furthermore, the effect of the additives was not limited to the *Trichoderma* isolate but may have promoted microflora competition on the wood substrate as indicated by the higher diversity indices from wounds treated with conidial suspension 2 than from wounds treated with suspension 1. Previously Simon and Sivasithamparam (1988) observed the inhibition of *Trichoderma* spp. by different bacteria in dual culture tests. Naár and Kecskes (1998) demonstrated that in several *in vitro* tests the bacteria *Clavibacter michiganese* and *Pseudosomas syringae* caused significant inhibition of *Trichoderma* spp. by the production and excretion of antibiotic substances. Lutz et al. (2003) observed a negative influence and a reduction of antagonistic activity by the fungi *Fusarium culmorum* and *F. graminearum*.

Carriers of inocula are inert ingredients in the sense that they do not have disease control capacity; however they can profoundly affect time of germination as well as viability of conidia (Fravel et al., 1998). In the present study, the conidia of T-15603.1 enriched by additives in a humidity storing gel formulation providing a constant water availability and thus remained the conidia viable and effective, as indicated by the significant ($P < 0.001$) highest re-isolation rate from wounds treated with the conidial suspension 3. In addition conidial adhesion to the wood substrate was improved with the use of a gel as carrier (Batta, 2004; Jayaraj et al., 2006). The increased viability and adhesion enhanced the establishment of the biocontrol agent and thus improved its strength of competition as shown by

the lowest diversity indices from wounds inoculated with conidial suspension 3. In particular the low E index 0.242 (high portion of the antagonist on total number of individuals or groups) shows that T-15603.1 was the dominant species on the wood substrate.

Isolation rate of ubiquitous *Trichoderma* spp. from the untreated control wounds was consistently low (4.5%). There is no explanation for this at present other than speculation that on urban sites, the presence of *Trichoderma* spp. and its inoculum potential could be limited due to the absence of organic matter, which is essential for growth, survival and sporulation. More detailed studies are needed to confirm this. The highest diversity indices were detected from control wounds. In particular the low E index (0.846) is an indication that on the untreated substrate natural succession occurred without influence of the competitive T-15603.1.

Although formulation of the conidia and abiotic factors, especially humidity, influenced the establishment of the biocontrol agent, the wood substrate appeared also to be a decisive factor. The re-isolation rate was significantly ($P < 0.05$) reduced on heartwood. Shigo and Hills (1973) investigated the specific characteristics of heartwood from several tree species and they described heartwood as a substrate with low wood moisture and with fungistatic properties, which hampers colonization by microorganism. Thus, the conidial viability and thereby the biocontrol efficacy of T-15603.1 is limited on large wound dimensions because of an increased ratio of heartwood to sapwood.

Successful infection and colonization of untreated wounds by wood decay fungi depends on the ability to overcome host barriers in the wood and to circumvent and/or degrade phenolic compounds (Schwarze et al., 1999; Schwarze and Ferner, 2003). *Inonotus hispidus* and *Polyporus squamosus* are classified as wound parasites and as such are able to infect and colonize small wounds (McCracken and Toole, 1974; Schwarze et al., 1999). The ability of *G. adspersum* to degrade polyphenolic deposits in reaction zones was recently demonstrated (Schwarze and Ferner, 2003) and explains the high infection rate (78.7%) of the untreated wounds within 30 months. Pre-treatment of the wounds with T-15603.1 resulted in strong protection against wood decay fungi, particularly *I. hispidus* and *G. adspersum* (90%). This is in good agreement with the results obtained here in laboratory tests that showed high sensitivity of both basidiomycetes against T-15603.1. A low effect (62.4%) was measured with *P. squamosus*, which is also in good agreement with results obtained in the *in vitro* studies. The active mechanisms of antagonism in wood substrate are determined by fast growth, mycoparasitism and antibiosis. A further passive effect of T-15603.1 could be related in maintaining the tree's defense boundaries. Smith et al., 1981 postulated that *T. harzianum* can tolerate high levels of phenols produced by the host in response to colonization without the need to modify compounds. Other fungi such as *Phialophora melinii* (Nannf.) Cont., which is also tolerant of fungistatic

substances, may reduce the efficiency of defense boundaries by metabolizing large amounts of polyphenols (Smith et al., 1981). Inhibition and exclusion of such fungi may maintain high levels of polyphenols thus preventing colonization by decay fungi which are sensitive to fungistatic substances. Whether the treatment with T-15603.1 helped in maintaining defense boundaries could not definitely be demonstrated, but the present work provides strong evidence that the inoculation of T-15603.1 not only inhibited decay fungi from colonizing tree wounds but also many other fungi as demonstrated by the diversity indices.

Accurate identification and the ability to specifically identify the isolate T-15603.1 was critical for interpretation of the results, especially considering the high degree of misidentification of *Trichoderma* species in the literature (Kulling et al., 2001). In the present work, accurate identification was efficiently performed by RAPD (Castle et al., 1998). Results obtained from *in vitro* studies were helpful in eliminating non-performing isolates from the screening tests but were not sufficient due to the fact that *in vitro* assays not completely mimic all ecological and endemic factors. Factors such as water movement, competition with the indigenous microflora are impossible to reproduce in the laboratory. For this reason field studies are essential to test the selected biocontrol agent under natural conditions. *In vitro* and field results demonstrated that the *Trichoderma* strain T-15603.1 can be successful when applied as biological wound treatment against *I. hispidus* and *G. adspersum* on urban sites. But there is still a need to optimize the formulation of conidia to improve the establishment and therefore the biocontrol efficacy, particularly on pruning wounds with high heartwood to sapwood ratios. In addition, it is possible that isolates exist that are more effective and persistent than T-15603.1. Therefore further screening trials of competitive strains against a range of common wood decay fungi should be undertaken. The application of T-15603.1 on tree wounds cannot completely inhibit colonization by all wood decay fungi. In addition genotype and virulence of wood decay fungi may vary from those reported in this work. A strategy to enhance the effect of a biological wound treatment could be the use of a mixture of biocontrol strains, which may provide a greater protection under different environmental conditions than the application of individual biocontrol strains (Meyer and Roberts, 2002).

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