Members of the Trichoderma genus are known as soil-borne fungi, fast-growing in culture and producing numerous pigmented spores. They occur worldwide and are commonly associated with root, soil, and plant debris. Trichoderma species have long been recognized as biological agents for controlling plant diseases. Past research indicates that Trichoderma can parasitize fungal pathogens and produce antibiotics. More recent research indicates that certain strains of Trichoderma can induce systemic acquired resistance in plants. Several commercial biological products based on Trichoderma species are manufactured and marketed worldwide for use against a wide range of plant pathogens. Currently, there is little knowledge on the effectiveness of biological control agents of wood-decay fungi that colonize urban trees. Studies in Europe, Asia, and Australia indicate that, after careful selection of native and highly antagonistic Trichoderma strains, a range of wood-decay fungi can be used to successfully control or eradicate the casual decay fungus from pruning wounds and wood debris within the soil.

Francis W.M.R. Schwarze, Ph.D., Swiss Federal Laboratories for Materials Science and Technology

Dr. Francis Schwarze is an officially appointed and attested expert on wood decay of urban trees, concentrating on research into wood-decay fungi, fungus–host interactions, and the use of fungi for beneficial purposes (e.g., improving the acoustic properties of wood for violins or biological control). He is a member of the British Mycological Society and head of the Research Group Bio-engineered Wood, Applied Wood Materials Laboratory, EMPA, Swiss Federal Laboratories for Materials Science and Technology. Dr. Schwarze has made highly significant contributions to our fundamental understanding of wood decay by fungi. He has applied the knowledge for the benefit of such important wood science and technology areas as permeability enhancement of heartwoods and refractory timbers, detection of tree rings, performance evaluation of thermo-mechanically densified wood, enhancement of acoustic properties of violins, and biocontrol of decay fungi affecting urban tree health. Many areas of his work have been regarded as path-breaking research and have been published in high impact international journals, such as New Phytologist and Nature Biotechnology, attracting attention of such high profile journals as Nature for commentaries.
Biological control of wood decay fungi with *Trichoderma* spp.

Prof. Dr. Francis W.M.R. Schwarze
Applied Wood Science
Bio-engineered Wood

Friday, December 4, 2015

Biological control of pests and diseases

- Green lacewing fly (*Chrysoperla carnea*)
- Convergent lady beetle (*Hippodamia convergens*)
- *Aphidius ervi* (Parasitic wasp)
Advantages and disadvantages of biological control

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selectivity, it does not intensify or create new pest problems</td>
<td>Control is slow</td>
</tr>
<tr>
<td>No manufacturing of new chemicals</td>
<td>It will not exterminate broad spectrum of pathogens</td>
</tr>
<tr>
<td>The pest is unable (or very slow) to develop a resistance</td>
<td>It is often unpredictable</td>
</tr>
<tr>
<td>Control is self perpetuating</td>
<td>It is difficult and expensive to develop and supply</td>
</tr>
<tr>
<td></td>
<td>It requires expert supervision.</td>
</tr>
</tbody>
</table>


What is *Trichoderma*?

*Trichoderma* spp. are free-living fungi that are common in soil and root ecosystems. Recent discoveries show *Trichoderma* spp. are opportunistic, avirulent plant symbionts, as well as being parasites of other fungi.


Taxonomy of *Trichoderma*

**Mycota**

<table>
<thead>
<tr>
<th>Division</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascomycota</td>
<td>Sordariomycetes</td>
<td>Hypocreales</td>
<td>Hypocreaceae</td>
</tr>
</tbody>
</table>

Important species used for biocontrol:

*Trichoderma atroviride*
*Trichoderma harzianum* T22
*Trichoderma hermatum*  
*Trichoderma virens*
Microscopic features of *Trichoderma*

Septate hyaline hyphae. Conidiophores are hyaline, branched, phialides are hyaline, flask-shaped, and inflated at the base.

The colour of the conidia is mostly green. *Trichoderma* spp. may also produce chlamydospores.

Mechanisms by which *Trichoderma* spp. function

- Mycoparasitism
- Antibiosis
- Competition for nutrients or space
- Tolerance to stress through enhanced root and plant development
- Solubilization and sequestration of inorganic nutrients
- Systemic acquired resistance (SAR)
- Inactivation of the pathogen’s enzymes


*Mycoparasitism*

*Trichoderma* spp. grow tropically toward hyphae of other fungi, coil about them in a lectin-mediated reaction, and degrade cell walls of the target fungi.

Antibiosis

Trichoderma spp. may release antibiotics (e.g., gliotoxin and viridin) and/or VOCs (volatile organic compounds) that slows down or kills a pathogen in the vicinity of such a product.


Competition for nutrients or space

Trichoderma spp. often grow faster or use food sources more efficiently than the pathogen, thereby crowding out the pathogen and taking over. The pathogen can not compete with the biocontrol agent.


Growth rates of Trichoderma and wood decay fungi

Growth rates of Trichoderma and wood decay fungi.

ø 5fold stronger growth rate!

Tolerance to stress through enhanced root and plant development

Enhanced root development from field-grown corn and soyabean (Harman G.E. 2000)

Improved survival of pepper plants in the field (Harman, G.E. 2000)

Systemic acquired resistance (SAR)

Some Trichoderma strains clearly are potent inducers of SAR-like responses. When inoculated onto roots or leaves they can provide control of disease caused by Botrytis cinerea on leaves spatially separated from the site of application of the biocontrol agent.

Model of induced resistance in tomato.


Inactivation of the pathogen’s enzymes

When applied to leaves Trichoderma spp. produce a serine protease that is capable of degrading the pathogens plant cell wall degrading enzymes and thereby reducing the ability of the pathogen to infect the plant.

Effect of crude protease from cultures of Trichoderma harzianum T39 (*) and NCIM1185 (●) on the germination rate of conidia of Botrytis cinerea (left). Control (◊). Elad & Kapat (1999).
Solubilization and sequestration of inorganic nutrients

Trichoderma spp. can increase iron availability at pH values above 6.5.

Appearance of Cantharanthus cv. Parasol plants grown in planting mix at about pH 7 in the absence and presence of T-22.Courtesy George Elliot.

Bumble bees as bee-delivery technique for the dispersal of Trichoderma

- Insertion of Trichoderma conidia into the bee hive
- Bees deposit conidia on flowers they visit as they search for pollen and nectar
- Bee-delivery is twice as effective as spraying
- Strawberry yields improve by 20 -30 %.

Trichoderma is highly effective when applied to blossoms or fruits for control of Botrytis cinerea.

Treatment of pruning wounds

- Dujesiefken, 1992; 1995 - treatment with wound sealants not effective.
- Legislation, 1998 - wound treatment with chemicals not always legally permitted.
- Dubos & Ricard, 1974 - curative treatment with Trichoderma spp. against Chondrostereum purpureum.
- BINAB, 1976 - the first company to receive a registration for a biofungicide in the western world.
- Pettie & Shigo, 1975 - treatment of Acer rubrum wounds with Trichoderma viride resulted in a reduction in isolates of Hyphomycetes and Basidiomycetes.
- Pettie et. al., 1977 - treatment of Acer rubrum wounds with Trichoderma harzianum protected colonisation against basidiomycetes for two years.
- Smith, 1981 - Trichoderma spp. counteracted modification of polyphenols by suppressing the growth of Hyphomycetes.
- Mercer & Kirk, 1982 - Wound treatment with Trichoderma spp. partly protected host from infection by basidiomycetes.
- Lonsdale, 1992 - Successful wound treatment with Trichoderma spp. against Chondrostereum purpureum. Even five years after treatment Trichoderma was present in the wood.
Field studies to evaluate the efficiency of *Trichoderma* spp. for biological control against wound pathogens

- Treatment of pruning wounds of a range of hosts on different sites with conidial suspensions of T-15603.1
- Reisolation and monitoring of T-15603.1 in the field
- Identification of T-15603.1
- Impact of T-15603.1 on wound wood formation and extent of dysfunctional wood.


Objectives of the investigations

- To evaluate the potential of different *Trichoderma* spp. as biocontrol agents
- To identify a competitive species that can be used for the treatment of pruning wounds on urban trees against colonisation by wood decay fungi
- To establish a method that promotes colonisation and survival of a selected *Trichoderma* isolate in pruning wounds.


*Trichoderma* isolates and wood decay fungi

<table>
<thead>
<tr>
<th><em>Trichoderma</em> spp.</th>
<th>Source and origin</th>
<th>Isolate-No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichoderma atroviride</em></td>
<td>AMI 216201.3</td>
<td>IMI 211631.3</td>
</tr>
<tr>
<td><em>Trichoderma albolentum</em></td>
<td>AMBI 292921.2</td>
<td>IMI 206061.2</td>
</tr>
<tr>
<td><em>Trichoderma atroviride</em></td>
<td>IPK 125.65</td>
<td>IMI 206060.2</td>
</tr>
<tr>
<td><em>Trichoderma atroviride</em></td>
<td>IPK 152.65</td>
<td>IMI 206063.2</td>
</tr>
<tr>
<td><em>Trichoderma atroviride</em></td>
<td>IPK 157.65</td>
<td>IMI 206067.2</td>
</tr>
<tr>
<td><em>Trichoderma harzianum</em></td>
<td>IMI 206039.3</td>
<td>IMI 206040.3</td>
</tr>
<tr>
<td><em>Trichoderma. polysporum</em></td>
<td>IMI 206040.3</td>
<td>IMI 206041.3</td>
</tr>
</tbody>
</table>

¹ Isolates from the Forest Botany, University of Freiburg.
² Isolates from Centraalbureau voor Schimmelcultures, the Netherlands.
³ BINAB Bio-Innovation AB, Sweden.
Classification of the degree of antagonism of different *Trichoderma* spp.

<table>
<thead>
<tr>
<th>In vitro studies</th>
<th>Index of dominance (ID)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rates</td>
<td>ID I</td>
<td>1 – 5</td>
</tr>
<tr>
<td>Germination of conidia</td>
<td>ID II</td>
<td>1 – 5</td>
</tr>
<tr>
<td>Affect of VOCs</td>
<td>ID III</td>
<td>1 – 5</td>
</tr>
<tr>
<td>Dual culture tests</td>
<td>ID IV</td>
<td>1 – 5</td>
</tr>
<tr>
<td>Interaction studies in wood</td>
<td>ID V</td>
<td>1 – 5</td>
</tr>
</tbody>
</table>

\[ \text{Index (ID-Index)} = \frac{\sum \text{ID}}{\sum \text{tests}} \]

Mean growth rate of the *Trichoderma* isolates under different conditions (mm d\(^{-1}\), ±SE)

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>MEA</th>
<th>LNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>2.0 ± 0.15</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>8.9 ± 0.21</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>6.4 ± 0.15, 18.8 ± 0.38</td>
</tr>
<tr>
<td>30</td>
<td>3.6 ± 0.23, 12.9 ± 0.17</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Water activity aw</th>
<th>Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.892</td>
<td>5 0 0 0 0</td>
</tr>
<tr>
<td>0.995</td>
<td>10 0 7.7 ± 2.8, 10.3 ± 3.3, 44.5 ± 21.8</td>
</tr>
<tr>
<td>0.996</td>
<td>15 0 14.8 ± 6.2, 22.7 ± 7.1, 95.8 ± 13.5, 99.2 ± 21.2</td>
</tr>
</tbody>
</table>

Inhibition of radial growth (%) of wood decay fungi by VOCs produced by *Trichoderma* spp

Classification of mycelial-interactions in dual culture tests

Legend:
0 = no antagonism
1 = Trichoderma overgrows mycelium of target fungus (mycoparasitism)
-1 = Target fungus overgrows mycelium of Trichoderma

Legend:
0 = No antagonism
1 = Antibiosis
2 = Trichoderma overgrows mycelium of target fungus (mycoparasitism)
3 = Target fungus overgrows mycelium of Trichoderma

Classification of mycelial-interactions in dual culture tests

Dual culture tests

Trichoderma / Ganoderma adspersum

Trichoderma / Polyporus squamosus

Classification of the degree of mycoparasitism of different Trichoderma spp. on MEA, ± SE

<table>
<thead>
<tr>
<th>MEA</th>
<th>T-1593.1</th>
<th>T-351.63</th>
<th>T-356.92</th>
<th>T-655.65</th>
<th>T-356.93</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. implicis</td>
<td>2.4 ± 0.14 (100%)</td>
<td>2.9 ± 0.90 (100%)</td>
<td>2.4 ± 0.55 (100%)</td>
<td>2.3 ± 0.77 (100%)</td>
<td>2.5 ± 0.89 (100%)</td>
</tr>
<tr>
<td>G. adspersum</td>
<td>3.0 ± 0.16 (100%)</td>
<td>2.5 ± 0.16 (100%)</td>
<td>2.9 ± 0.12 (100%)</td>
<td>2.4 ± 0.89 (100%)</td>
<td>2.7 ± 0.99 (100%)</td>
</tr>
<tr>
<td>G. quercina</td>
<td>2.3 ± 0.11 (83%)</td>
<td>2.4 ± 0.23 (100%)</td>
<td>1.9 ± 0.21 (87%)</td>
<td>1.8 ± 0.33 (94%)</td>
<td>0.3 ± 0.06 (90%)</td>
</tr>
<tr>
<td>G. abutschii</td>
<td>3.0 ± 0.38 (100%)</td>
<td>2.6 ± 0.31 (100%)</td>
<td>2.6 ± 0.33 (100%)</td>
<td>2.6 ± 0.42 (100%)</td>
<td>2.6 ± 0.56 (100%)</td>
</tr>
<tr>
<td>P. squamosus</td>
<td>3.2 ± 0.58 (83%)</td>
<td>2.9 ± 0.12 (100%)</td>
<td>2.4 ± 0.66 (85%)</td>
<td>1.7 ± 0.17 (93%)</td>
<td>1.9 ± 1.05 (100%)</td>
</tr>
</tbody>
</table>

** 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.

* 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.
Evaluation of antagonistic activity in wood

Interaction studies in wood blocks

Ganoderma adspersum / Trichoderma

Charcoal stump rot

Kretzschmania deusta / Trichoderma

Interaction studies in wood blocks

Polyporus squamosus / Trichoderma


Field studies

I. Wound treatment:
Spring / summer 2003
- 9 pruning wounds on 37 trees in Strasbourg and on 81 trees in Ludwigshafen, BASF AG were treated with conidial suspensions of T-15603.1

II. Infection trials with T-15603.1 and wood decay fungi:
- 9 pruning wounds on 78 trees were treated with T-15603.1 at a site in Freiburg. Three weeks after treatment wounds were additional inoculated with Inonotus hispidus, Ganoderma adspersum and Polyporus squamosus.

Index of dominance ID

<table>
<thead>
<tr>
<th>T-15603.1</th>
<th>T-15603.2</th>
<th>T-386.6</th>
<th>T-360.65</th>
<th>T-136.65</th>
<th>ID -indices</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dual culture tests</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-4-4-4-5</td>
<td>2-2-1-1-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOC's tests</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-2-4-3-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Interactions wood growth rates</td>
<td></td>
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<tr>
<td>4-4-3-3-5</td>
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<td></td>
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<tr>
<td>Germination rates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-4-3-3-2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean ID-value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.6 (a)</td>
<td>3.6 (a)</td>
<td>3.6 (a)</td>
<td>3.6 (a)</td>
<td>2.2 (c)</td>
<td></td>
</tr>
</tbody>
</table>

ID-value 0-1 = no antagonistic potential; ID-value 1-2 = weak antagonistic potential; ID-value 2-3 = moderate antagonistic potential; ID-value 3-4 = high antagonistic potential; ID-value 4-5 = very high antagonistic potential.
Range and number of tree species inoculated with T-15603.1

<table>
<thead>
<tr>
<th>Tree Species</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platanus × hispanica Moonch.</td>
<td>1</td>
</tr>
<tr>
<td>Acer pseudoplatanus L.</td>
<td>40</td>
</tr>
<tr>
<td>Tilia cordata L.</td>
<td>24</td>
</tr>
<tr>
<td>Populus nigra L.</td>
<td>16</td>
</tr>
<tr>
<td>Quercus spp. L.</td>
<td>16</td>
</tr>
<tr>
<td>Robinia pseudacacia L.</td>
<td>9</td>
</tr>
</tbody>
</table>

Total trees: 196
Total wounds: 1764


Treatment of pruning wounds with T-15603.1

Method A: Suspension [CFU: 10^5 conidia/ml]
Method B: Suspension [CFU: 10^7 conidia/ml + 0.2% glucose + 0.1% urea]
Method C: Suspension [CFU: 10^7 conidia/ml + 0.2% glucose + 0.1% urea + 0.4% Luquasorb® (Sodium polyacrylate)] *
Method K: Control – no treatment

*For method C a Hydrogel Luquasorb® 100 30 (BASF AG) was used as a carrier substance.

At the site in Strasbourg the Hydrogel Luquasorb® was not applied. For this reason an alternative method was used:
Method CS: Suspension [CFU: 10^5 conidia/ml + 0.5% glucose + 0.2% urea]


Field studies

Experiment I: Treatment of pruning wounds:

Tree spp.
Climate data
Ratio of sap- and heartwood
Wound dimensions
Wound occlusion
Discolouration

Measurement of wound occlusion

Reisolation of *Trichoderma atroviride* 15603.1 from pruning wounds

Extent of wood discolouration developing from pruning wounds

* a, b denote significant differences (P < 0.05) of wound wood formation for tree species. C: 1<30%; C: 31-60%; C: >60%.
Identification of *Trichoderma atroviride* 15603.1 with RAPD-PCR

Lane 1 = applied strain T-15603.1 (reference); lanes 2-6 = *Trichoderma* isolated from the treated pruning wounds after 30 months; lane 7 = *Trichoderma virens*; lane 8 = *Trichoderma fasciculatum*.

Reisolation results of T-15603.1 at the site in Ludwigshafen

Symbols with different letters indicate significant (p ≤ 0.05) differences in reisolation rates according to Ryan-Einot-Gabriel-Welsch-Test (REWGQ).

Reisolation rate of T-15603.1 from sap- and heartwood
Reisolation rate of T-15603.1 in relation to wound size

Reduction of infection rate by wood decay fungi after wound treatment with T-15603.1

Trichoderma Research Singapore

Pruning wounds as infection points for wood decay fungi

- Stimulates resource allocation among growth, defense, reproduction and storage
- harbours minimal structural and fast-infecting capacity of branches
- results in necrosis, tissue necrosis, and carbon dioxide

Identify causal fungal interactions biological control

- Healthy, living trees inoculated with wood decay fungi
- One year incubation period
- To be felled, harvested, and dissected to evaluate infection severity

Re-isolation rates for the respective Trichoderma strains applied to each of the two tree species

<table>
<thead>
<tr>
<th>Control</th>
<th>Hydrogel suspension</th>
<th>Water suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.0%</td>
<td>14.0%</td>
<td>15.0%</td>
</tr>
<tr>
<td>80.6%</td>
<td>69.3%</td>
<td>62.4%</td>
</tr>
<tr>
<td>82.1%</td>
<td>69.4%</td>
<td>65.8%</td>
</tr>
<tr>
<td>13.6%</td>
<td>12.7%</td>
<td>11.78%</td>
</tr>
<tr>
<td>46.3%</td>
<td>31.95%</td>
<td>29.2%</td>
</tr>
<tr>
<td>29.2%</td>
<td>25.98%</td>
<td>25.98%</td>
</tr>
</tbody>
</table>

1. Treat fresh pruning wounds with Trichoderma conidial suspensions
2. Monitor colonization and persistence of Trichoderma on pruning wounds
3. Evaluate the effect of Trichoderma on wound occlusion and infection rates.

Phellinus noxius (Brown root rot)

Susceptibility

The USDA-ARS Systematic Botany and Mycology Laboratory currently lists 223 host species. Some of the most notable include mahogany, teak, rubber, oil palm, tea, coffee, and cacao as well as a variety of fruit, nut, and ornamental trees.

Hosts of *Phellinus noxius*

- Araucaria bidwillii
- Delonix regia
- Ficus benjamina
- Jacaranda microphylla

Conditions favoring disease of *Phellinus noxius*

Chang (1996) found that no *Phellinus noxius* was recovered from soils containing infested root debris after one month of flooding.

Disease incidence of *Phellinus noxius* on different soil types

**Biocontrol of *Phellinus noxius* (Brown root rot) in Brisbane, Australia**

Keith Foster, Senior Coordinator Arboriculture, Brisbane City Council.


**Biocontrol of *Phellinus noxius* (Brown root rot)**

Keith Foster, Brisbane City Council:

"A great outcome, I would like to thank you so much for your help, the trees that we have used the *Trichoderma* on are all recovering. The Banyan fig at Shorncliffe prior to introducing the *Trichoderma* had little leaf in the canopy and what leaf was left were yellow, now it has a new canopy of leaf with a dark green color. Amazing.

Once again thank you, without you help, we would not have been in a situation where we can manage the diseased trees, they would have been lost."
Biocontrol of *Phellinus noxius* in Hong Kong, China


Wood decay triangle

<table>
<thead>
<tr>
<th>PATHOGEN</th>
<th>HOST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of Inoculum</td>
<td>Total of conditions favouring susceptibility</td>
</tr>
<tr>
<td>ENVIRONMENT</td>
<td>HOST</td>
</tr>
<tr>
<td>Total of conditions favouring disease</td>
<td>Amount of Decay</td>
</tr>
</tbody>
</table>

Agrios (1988) stated that the three components of the decay triangle could be quantified. This would enable the area of the triangle to represent the amount of decay.

Biocontrol of *Phellinus torulosa* in Italy
29.9.2014 Two weeks after first treatment

16.10.2014 One month after the first treatment

12.2.2015 Five months after first treatment
Biocontrol methods may be beneficial for veteran trees growing on sites with low microbial activity.

Circumstantial evidence suggests that carefully selected antagonists can be applied against wood decay fungi that are weak competitors.

Trichoderma spp., can reduce decomposition rates of opportunistic wood decay fungi in trees and induce SAR.
Evaluation of an antagonistic *Trichoderma* strain for reducing the rate of wood decomposition by the white rot fungus *Phellinus noxius*

Francis W.M.R. Schwarze\textsuperscript{a,}, Frederick Jauss \textsuperscript{a}, Chris Spencer \textsuperscript{b}, Craig Hallam \textsuperscript{b}, Mark Schubert \textsuperscript{a}

\textsuperscript{a} EMPA, Swiss Federal Laboratories for Materials Science and Technology, Wood Laboratory, Section Wood Protection and Biotechnology, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland

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**Highlights**

- Antagonism of *Trichoderma* species against *Phellinus noxius* varied in the *in vitro* studies.
- Weight losses by *P. noxius* were higher in angiospermous than gymnospermous wood.
- Biocontrol of *P. noxius* depends on the specific *Trichoderma* strain and its host.

**Graphical Abstract**

![Graphical Abstract](image)

**Abstract**

The objective of these *in vitro* studies was to identify a *Trichoderma* strain that reduces the rate of wood decomposition by the white rot fungus *Phellinus noxius* and *Ganoderma australe*. For this purpose, dual culture and interaction tests in wood blocks of three hardwoods, *Delonix regia*, *Ficus benjamina*, *Jacaranda mimosifolia*, and one softwood, *Araucaria bidwillii*, as well as investigations of fungal growth under different environmental conditions, were performed. The effect of *Trichoderma ghanense*, two strains of *T. harzianum* and *T. reesei* on wood colonization and decomposition by four *P. noxius* strains and *G. australe* were quantitatively analyzed by measuring the dry weight loss of wood. All *Trichoderma* species and wood-decay fungi showed optimum growth at a mean temperature of 25–35 °C and a high water activity \(a_w\) of 0.998. At 35 °C and \(a_w\) 0.928, no growth was recorded for any of the wood-decay fungi after 1 week, whereas most *Trichoderma* species were still actively growing. The different *Trichoderma* species all showed an antagonistic potential against *P. noxius* in the *in vitro* studies. The species of wood-decay fungi showed significant differences in their sensitivity when challenged by the volatile organic compounds (VOCs) of *Trichoderma* species. Reduction in the rate of wood decomposition by different *Trichoderma* species against all wood-decay fungi varied strongly according to the specific plant host. *T. harzianum* 121009 and *T. atroviride* 15603.1 showed the highest reduction in weight losses. *P. noxius* strongly decomposed untreated and pretreated wood of *D. regia*, whereas weight losses of *F. benjamina* and *J. mimosifolia* pretreated with *Trichoderma* strains were significantly lower. Weight losses by *G. australe* were significantly reduced for *A. bidwillii*, *D. regia* and *F. benjamina* by all *Trichoderma* species, but no affect was recorded for *J. mimosifolia*. The *in vitro* studies show that only after careful monitoring (i.e. selecting the appropriate strain for the target pathogen and its niche (wood species) can *Trichoderma* species be used to significantly reduce the growth and rate of wood decomposition by different *P. noxius* strains.

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1. Introduction

Species of the genus *Trichoderma* are ubiquitous in soils. Since Weindling (1932) recognized the antagonistic effect of *Trichoderma* species against plant pathogens, several have been extensively studied as biological control agents against fungal pathogens (Chet, 1990; Chet et al., 1998; Harman et al., 2004; Howell, 1998). As potential biocontrol agents, *Trichoderma* species have the following advantages: they grow on, but do not occur on, most organic matter, sporulate readily in culture and, in natural conditions, can act as either secondary antagonists or primary colonizers (Alabouvette et al., 2006; Highley, 1997; Holdenerider and Greig, 1998; Schoeman et al., 1999). Furthermore, some *Trichoderma* species survive as chlamydospores under unfavourable conditions and are fairly resistant to common fungicides and herbicides (Sariah, 2003).

Much of the biological control research in the tropics has focused on the development of strains of *Trichoderma* species (subdivision: Ascomycota) that show antagonistic activity against fungal root fungal pathogens (Harman et al., 2004; Prasad and Naik, 2002; Raziq and Fox, 2006; Sariah, 2003; Sariah et al., 2005; Susanto et al., 2005; Widyastuti, 2006). Soepena et al. (2000) successfully formulated a biofungicide comprising *Trichoderma koningii* Oud. isolate Marilhat (MR14) to manage basal stem rot in *Elaeis guineensis* (Jacq.) (oil palm) caused by *Ganoderma orbiforme* (Fr.) Ryvarden (= *G. boninense* Pat.). This pathogen is recognized as the single major disease constraint to sustainable production of oil palm throughout Asia (Ariffin et al., 2000; Durand-Gasselin et al., 2005; Flood et al., 2000; Paterson et al., 2000; Singh, 1991; Turner, 1981). In in vitro experiments, the growth of two unknown *Ganoderma* species, previously isolated from diseased *Acacia mangium* in Indonesia, were shown to be strongly inhibited by *T. koningii*, *T. harzianum* and *T. reesei* (Widyastuti, 2006). In field experiments carried out at different locations in France and Germany, a total of 159 angiospermous trees and 1431 wounds on six different species (*Platanus × hispanica* Miller ex Münchh., *Acer pseudoplatanus* L., *Tilia platyphyllos* Scop., *Populus nigra* L., *Quercus rubra* L., *Robinia pseudoacacia* L.) were treated with different conidial suspensions of *T. atroviride* strain T-15603.1, which has been shown to have high biocontrol efficacy against several wood-decay fungi (Schubert et al., 2008a–c), with four Australian native *Trichoderma* strains (*T. ghanense*, *T. reesi*, and two strains of *T. harzianum*).

2. Materials and methods

2.1. Micro-morphological and molecular identification

All cultures were identified microscopically (Bissett, 1984; 1991a–c; 1992; Gams and Bissett, 1988; Rifai, 1969) and additionally the internal transcribed spacer (ITS) 1-5.8S-ITS2 region of the rDNA was amplified and sequenced for each strain (Schubert, 2006). The origins of the *Trichoderma* species and wood-decay basidiomycetes are provided in Table 1. All cultures were maintained on 2% malt extract agar (MEA) at 4(±1)°C. For further studies, Petri dishes with MEA were inoculated with 5 mm diameter agar plug cut from the growing edge of colonies of the strains and stored in the dark at 25(±1)°C and 70% ambient relative humidity. The sequences were deposited in the EMBL Data Bank (Table 1).

2.2. Growth of *Trichoderma* species and wood-decay fungi under different conditions

The effects of temperature (20°C, 25°C, 30°C, 35°C and 35°C) and water activity (a_w: 0.928, 0.955, 0.978, 0.998) on hyphal growth were monitored on 2% MEA. All agar plates (90 mm) were inoculated centrally with a 5-mm disc of the respective *Trichoderma* species and wood-decay fungi taken from the margin of growing cultures and incubated at 25(±1)°C and 70% relative humidity. For each experimental treatment (a_w and temperature), 10 replicates were performed. The growth rate (mm/day) was determined by colony diameter measurements carried out along two perpendicular axes after 24 h (Schubert et al., 2009). The a_w of the substrate was controlled by the addition of appropriate weights of the non-ionic solute, glycerol, prior to autoclaving (Dallyn, 1978).
2.3. Inhibitory effects of volatile compounds produced by Trichoderma species on wood-decay fungi

The effect on wood-decay fungi of volatile organic compounds (VOCs) produced by Trichoderma strains was evaluated with the following techniques as described by Dennis and Webster (1971). Trichoderma strains were centrally inoculated onto 2% MEA by placing 5-mm discs taken from the margin of 7-day-old cultures and then incubating the plates at 25°C ± 1°C and 70% relative humidity for 3 weeks. MEA plates were inoculated centrally with 5-mm discs of the wood-decay fungi and then the top of each plate was replaced with the bottom of a Trichoderma-inoculated plate. Replicates without Trichoderma species were used as the control. Ten replicates were maintained for each treatment. The pairs of Petri dishes were fixed and sealed together with Para film and incubated at 25°C ± 1°C and 70% relative humidity. The diameter of the wood-decay fungi colonies was measured after an incubation period of 7 days and the inhibition of mycelial growth was calculated.

2.4. Dual culture and interaction tests on wood

Mycoparasitism of all Trichoderma strains against the selected wood-decay fungi was assessed in dual culture according to the method of Schubert et al. (2006). The agar disc method was carried out on 2% MEA. Mycelial discs (5 mm) were removed from 1-week-old MEA cultures of each of the five wood-decay fungi and placed equidistantly at the margin of Petri dishes (90 mm) containing 2% MEA. These were incubated at 25°C ± 1°C and 70% relative humidity for 3–4 days. Next, discs (5 mm) were removed from the margins of actively growing 1-week-old cultures of the Trichoderma species and placed at opposite sides of the dish, and incubated in the dark at 25°C ± 1°C and 70% relative humidity for 4 weeks. Petri dishes without Trichoderma were used as the control. Twenty replicates were used for each experiment. Petri dishes were examined at regular intervals. The sporulation tufts and pustules of Trichoderma fungi were used as an indication of its activity (Naár and Keckes, 1998). In order to check whether the antagonist was able to overgrow and parasitize the challenged wood-decay fungi, three agar discs (5 mm) were removed from non-sporulating regions of the mycelium of the wood decay fungus and placed on a Trichoderma-selective medium (Askew and Laing, 1993). After 7 days of incubation at room temperature, discs were observed for Trichoderma colonies. Competition (mycoparasitism rate) was assessed as follows: 0 = no overgrowth; 1 = slow overgrowth; 2 = fast overgrowth; 3 = very fast overgrowth and deadlock of the wood-decay fungi. The ability of Trichoderma species to eliminate the wood-decay fungi (lethal effect) during the four week incubation period was evaluated by aseptically transferring 5-mm discs from test plates to MEA with 2 mL thiabendazole (2-(40-thiazolyl)-benzimidazole; Merck, Darmstadt, Germany; 0.46 mg dissolved in lactic acid). T-MEA suppresses the growth of Trichoderma species but allows growth of wood-decay fungi (Sieber, 1995). The lethal effect of Trichoderma species was expressed as a percentage of the wood-decay fungi which were eliminated. In addition, interaction tests in sap-wood blocks (Ave. 5 × 25 × 40 mm) of three hardwoods, Delonix regia (Boj. ex Hook.) Raf., Jacaranda mimosifolia D. Don, and Ficus benjamina L., and one softwood, Araucaria bidwillii (Molina) K. Koch, were performed as described by Schubert et al. (2008a). For studies of colonization behaviour, wood blocks were inoculated with a conidial suspension of Trichoderma species (colony-forming units: 10⁸/mL + 0.2% β-glucuron + 0.1% urea) and placed onto Petri dishes with 2% MEA. After the wood blocks were completely colonized by Trichoderma they were placed with their cross sections onto 2-week-old cultures of the wood-decay fungi and incubated in the dark at 25°C ± 1°C for 12 weeks. Untreated wood blocks served as the control. Twenty replicates were used for each experiment. Before incubation wood blocks were oven-dried at 105°C for 24 h to determine the wood dry weight. The decay tests were run for 12 weeks at ~25°C, after which the wood blocks were removed, cleaned and oven-dried for measuring dry weight losses (Schwarze and Fink, 1998).

2.5. Statistical analysis

Growth data were log-transformed and data that were expressed as percentages, such as the wood weight loss, were arc-sine-transformed prior to analysis (ANOVA) and back-transformed to numerical values for presentation (expressed as mean ± SE). Means were separated using Dunett’s test at significance levels of p < 0.05 and p < 0.0001. To compare the performance of the Trichoderma isolates with each other a Tukey’s HSD (Honestly Significant Difference) test (P < 0.05) was additionally performed. The statistical package used for all analyses was SPSS® (Version 17.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Growth under different conditions

The influence of temperature and αw on the mean growth rates of the Trichoderma species and wood decay fungi is shown in
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Tables 2 and 3, respectively. Generally, the growth rates of all Trichoderma strains increased with increasing aw (Table 2). T-4510 showed the strongest growth at a mean temperature of 35 °C and at the highest aw of 0.998 (Table 2). T-3967 and T-121009 showed an optimum growth at a mean temperature of 30 °C and at the highest aw of 0.998, whereas the European strains T-15603.1 and T-121009 showed optimum growth at a mean temperature of 25 °C and the highest aw of 0.998. Even at 35 °C and the lowest water activity (aw, 0.928), growth was recorded for some Trichoderma species. P. noxius strains 144, 169 and 178 showed optimum growth at a mean temperature of 25–30 °C and a high aw of 0.998 (Table 3). By contrast, P. noxius 133 showed optimum growth at a mean temperature of 30 °C and moderate aw of 0.978. At the lowest aw (0.928) and at 25 °C and 30 °C, growth was only limited, and at 35 °C growth of the P. noxius strains failed completely. G. australe showed optimum growth at a mean temperature of 30 °C and high aw of 0.998, but no growth was recorded at any temperature at the lowest aw (0.928).

### 3.2. Evaluation of antagonistic activity in dual cultures

During initial screening of the Trichoderma strains, a variety of reactions were recorded as a result of antagonism. Growth of all wood-decay basidiomycetes was inhibited by at least one of the Trichoderma strains. Contact between wood-decay fungi and Trichoderma species occurred, but the ability to overgrow and parasitize the mycelia of the wood-decay fungi was highly dependent on the antagonistic potential of each Trichoderma strain and the resistance of the challenged wood-decay fungus to antagonism (Table 4). The lethal effect of mycoparasitism by Trichoderma species was most prevalent for T. harzianum 121009. It showed a high antagonistic potential against three P. noxius strains and G. australe. P. noxius 178 showed moderate resistance to T. harzianum 121009. Trichoderma reesei 3967 revealed the weakest effect against all wood-decay fungi. The highest resistance of P. noxius to antagonism of Trichoderma species was recorded for strain 133. The highest susceptibility of P. noxius to antagonism of Trichoderma species was recorded for strain 169 (Table 4).

### 3.3. Inhibitory effects of volatile compounds produced by Trichoderma species on wood-decay fungi

The results revealed that after 6 days’ incubation, the VOCs produced by T. atroviride 15603.1 caused a significant (p < 0.0001) inhibition of growth of all P. noxius strains (Table 5). None of the VOCs from the Australian Trichoderma strains were effective in reducing pathogen growth except T-4428 that inhibited P. noxius 178. The weakest effect against the P. noxius strains was recorded for T. harzianum 121009. In the presence of VOCs, the growth of P. noxius strains was often enhanced. By contrast, the VOCs of T. atroviride 15603.1 and T. harzianum 121009 markedly inhibited the growth of G. australe. Among the Trichoderma species, with regard to the production and effect of VOCs, the wood-decay fungi differed significantly in their reaction. P. noxius strains 169 and 133 showed a strong sensitivity to the VOCs, whereas P. noxius 144 showed a lower sensitivity (Table 5).

### Table 2

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Water activity (aw)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.998</td>
</tr>
<tr>
<td>T. atroviride 15603.1</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>8.3 ± 1.3</td>
</tr>
<tr>
<td>25</td>
<td>10.6 ± 0.7</td>
</tr>
<tr>
<td>30</td>
<td>8.8 ± 0.5</td>
</tr>
<tr>
<td>35</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>T. ghanense 4510</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>9.0 ± 1.3</td>
</tr>
<tr>
<td>25</td>
<td>11.8 ± 2.9</td>
</tr>
<tr>
<td>30</td>
<td>16.1 ± 1.6</td>
</tr>
<tr>
<td>35</td>
<td>18.1 ± 1.6</td>
</tr>
<tr>
<td>T. harzianum 4428</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>8.6 ± 0.7</td>
</tr>
<tr>
<td>25</td>
<td>12.0 ± 0.6</td>
</tr>
<tr>
<td>30</td>
<td>8.1 ± 0.5</td>
</tr>
<tr>
<td>35</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>T. reesi 3967</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>10.7 ± 0.4</td>
</tr>
<tr>
<td>25</td>
<td>11.7 ± 3.1</td>
</tr>
<tr>
<td>30</td>
<td>13.3 ± 0.7</td>
</tr>
<tr>
<td>35</td>
<td>11.7 ± 0.4</td>
</tr>
<tr>
<td>T. harzianum 121009</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>13.0 ± 1.7</td>
</tr>
<tr>
<td>25</td>
<td>11.8 ± 1.9</td>
</tr>
<tr>
<td>30</td>
<td>14.4 ± 1.4</td>
</tr>
<tr>
<td>35</td>
<td>6.3 ± 0.8</td>
</tr>
</tbody>
</table>

For each experimental treatment (water activity aw and temperature), 10 replicates were performed. The growth rate (mm/day) was determined by colony diameter measurements carried out along two perpendicular axes after 24 h. The aw of the substrate was controlled by the addition of appropriate weights of the non-ionic solute, glycerol, prior to autoclaving. Data were analyzed by analysis of variance (ANOVA) to test the effect of aw and temperature on growth rate (mm/day). ± Standard deviation.
4.1. Growth under different conditions

The competitiveness of *Trichoderma* species is based on rapid growth (i.e., a decisive feature for antagonism) (Chet, 1990; Chet et al., 1998; Hjeljord and Tronson, 1998). Physical as well as chemical factors influence growth and germination, therefore knowledge of the optimal conditions for these factors, as well as the influence of ecological factors on the antagonist and the target pathogen, is essential for successful application in the field (Hjeljord and Tronson, 1998; Kredics et al., 2000, 2003).

In this study, the growth of the *Trichoderma* strains was affected by the environmental factors tested. Most *Trichoderma* strains showed optimum growth at a mean temperature of 25–30 °C and a high *a0* of 0.998. Even at 35 °C and the lowest *a0* (0.928) growth was recorded for some strains. The results have to be interpreted carefully because predicting the behaviour of *Trichoderma* species under specific conditions is complicated by the mutual effect of environmental parameters (Harman, 2006).

Growth of all *P. noxius* strains at different temperatures did not differ significantly. Optimum growth for most strains was recorded at 30 °C, which is in good agreement with the experimental findings of Ann et al. (2002), who showed that the optimum growth of *P. noxius* is at 30 °C, and that of Albrecht and Venette (2008), who defined the optimum growth range as approximately 25–31 °C. All of the *P. noxius* strains in this study were isolated from urban sites in Brisbane, Queensland, Australia. Interestingly, the optimum growth of the *P. noxius* strains correlates closely with a recent study on the local soil temperature. Prangnell and McGowan (2009) used a soil temperature calculation equation to calculate soil temperature at various depths in a cemetery located in Brisbane. Their study revealed that throughout the year the mean temperature at a soil depth of 1 m was approximately 28 °C. In the absence of vegetation cover, extreme temperatures of approximately 17 °C and 40 °C were measured. In soils with vegetation cover, only minor temperature fluctuations were reported (Prangnell and McGowan, 2009). The present study showed that the growth of different *P. noxius* strains does not appear to be strongly affected by different temperatures.
In comparison with the *P. noxius* strains, growth of most *Trichoderma* species was more rapid at the highest and lowest water activity values (0.998 and 0.928). At the lowest *a_w* (0.928) and at 25 °C and 30 °C, growth was minimal and at 35 °C the growth of the *P. noxius* strains failed completely.

The European species, *T. atroviride* 15603.1, that was used as a reference strain for reducing the rate of wood decay (Wood decay weight loss in %). Dunnett’s test.

**Table 6**

| Dry weight losses (in %) caused by *Phellinus noxius* and *Ganoderma australe* untreated controls and in wood blocks pretreated with different *Trichoderma* strains.

<table>
<thead>
<tr>
<th><em>T. atroviride</em> 15603.1</th>
<th><em>T. ghanense</em> 4510</th>
<th><em>T. harzianum</em> 4428</th>
<th><em>T. reesei</em> 3967</th>
<th><em>T. harzianum</em> 121009</th>
<th><em>G. australe</em> 121009</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><em>T. atroviride</em> 15603.1</td>
<td><em>T. ghanense</em> 4510</td>
<td><em>T. harzianum</em> 4428</td>
<td><em>T. reesei</em> 3967</td>
<td><em>T. harzianum</em> 121009</td>
</tr>
<tr>
<td>Control</td>
<td>34.78±17.02</td>
<td>13.01±15.17</td>
<td>23.05±7.40</td>
<td>13.61±4.35</td>
<td>24.05±10.42</td>
</tr>
<tr>
<td>Dry weight losses</td>
<td>21.34±13.36</td>
<td>14.65±7.94</td>
<td>18.07±8.24</td>
<td>20.15±2.04</td>
<td>23.62±5.71</td>
</tr>
<tr>
<td>(in %)</td>
<td>22.03±5.44</td>
<td>23.95±9.29</td>
<td>23.45±6.37</td>
<td>22.20±5.35</td>
<td>22.83±7.19</td>
</tr>
<tr>
<td>for wood blocks pretreated with different <em>Trichoderma</em> strains.</td>
<td>18.43±9.74</td>
<td>12.31±3.66</td>
<td>20.97±9.82</td>
<td>22.61±7.33</td>
<td>19.96±6.51</td>
</tr>
<tr>
<td><em>P. noxius</em> 133</td>
<td>3.49±1.98</td>
<td>4.42±3.83</td>
<td>4.24±1.38</td>
<td>3.64±2.79</td>
<td>4.42±4.38</td>
</tr>
<tr>
<td><em>P. noxius</em> 144</td>
<td>4.48±1.38</td>
<td>5.55±1.24</td>
<td>0.26±0.00</td>
<td>0.79±0.57</td>
<td>1.43±0.33</td>
</tr>
<tr>
<td><em>P. noxius</em> 169</td>
<td>5.49±1.38</td>
<td>4.24±1.38</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td><em>P. noxius</em> 178</td>
<td>4.49±1.38</td>
<td>5.55±1.24</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

Untreated wood blocks served as the control. Twenty replicates were used for each experiment. Before incubation wood blocks were oven-dried at 105 °C for 24 h to determine the wood dry weight. The decay tests were run for 12 weeks at ~25 °C, after which the wood blocks were removed, cleaned and oven-dried for measuring dry weight losses. Significant reduction of wood decay (wood weight loss in %).

In vitro tests conducted by Sivan and Chet, 1989 showed that two strains of *Trichoderma harzianum* failed to parasitize colonies of *Fusarium oxysporum* f. *vasinfectum* (G.F. Atk.) W.C. Snyder and H.N. Hansen and *Fusarium oxysporum* f. *melonis* W.C. Snyder and H.N. Hansen. However, these strains were strongly mycoparasitic on *Rhizoctonia solani* J.G. Kühn and *Pythium aphidermatum* (Edson) Fitzp. (Sivan and Chet, 1989). The study suggests that proteins in the cell walls of *F. oxysporum* may make these walls more resistant than those of *R. solani* to degradation by extracellular enzymes of *T. harzianum*. Savoie et al. (2000) studied interactions between *Lentinula edodes* (Berk.) Pegler and *Trichoderma* spp. and observed that there were great differences between two modified substrates for each strain. Similar experiments were performed with *T. harzianum* by Tokimoto and Komatsu (1979) who observed that carbon rich medium favours *L. edodes* while a nitrogen rich medium favours *Trichoderma* and that mycoparasitism could be reduced by controlling nutritional conditions. Giovannini et al. (2004) selected resistant strains of the wood decay fungi *Grifola frondosa* (Dicks.) Gray and *Fomitopsis pinicola* (Szw.) P. Karst. with a Teflon tubes confrontational method. Results showed great variations in behaviour between strains, but also for a given strain growing on different substrates, indicating the importance of trophic factors (Giovannini et al., 2004). Interestingly, in their experiments with *F. pinicola* they demonstrated that the substrate with the lower nitrogen content was more favourable for *Trichoderma* (Giovannini et al., 2004). In a previous study on mycoparasitism of a range of *Trichoderma* species against wood decay fungi, the white rot fungus *Polyporus*
angiospermous wood consists of approximately equal ratios of
Trichoderma species on wood-decay fungi

The spontaneous and soft rot (Schwarze, 2007). White rot is subdivided into simul-
mode of degradation of the woody cell wall: brown rot, white rot
wood
Fungal

Inhibitory effects of volatile compounds produced by Trichoderma
species on wood-decay fungi

Antibiosis was recognized and initially described by Weindling
(1932) and is defined as the production of secondary metabolites
that have an antimicrobial effect, even at low concentrations (Coo-
ney et al., 1997a,b; Galindo et al., 2004; Howell, 1998; Scarselletti
and Faull, 1994; Schirmböck et al., 1994; Wheatley et al., 1997).
The VOCs produced by T. atroviride 15603.1 strongly inhibited
growth of all P. noxius strains used in this study. Interestingly,
the VOCs produced by T. harzianum 121009 and T. ghanense 4510
appeared to stimulate the growth of P. noxius strains, however
the effect was not significant. The VOCs studies also indicated that
the antagonistic potential of Trichoderma species against P. noxius
varies from strain to strain, so for successful selection of a Tricho-
derma species for biological control of P. noxius these variations
in strain resistance have to be considered.

4.4. Interaction tests on wood

In order to assess the antagonistic potential of Trichoderma spe-
cies on its natural substrate, interaction studies were performed on
wood blocks. After 12 weeks' incubation, the Trichoderma species
failed to completely inhibit decomposition, as measured by dry
weight losses. This may be partly explained by the degradation of
readily accessible carbohydrates by Trichoderma species within
parenchymal cells and pits (Kubíček-Pranz, 1998). The inoculum
potential in turn is crucial for the invasiveness of pathogens (Red-
fern and Filip, 1991). Nevertheless, a significant reduction in dry
weight loss was induced after pretreatment of the wood with a
conidial suspension of Trichoderma species. Significant differences
between the species and strains of Trichoderma were evident.
Interestingly the antagonistic potential of different Trichoderma
species against P. noxius strains varied according to the specific
wood substrate. Thus, P. noxius strains showed a different degree
of adaptation to the wood. Phellinus noxius 169 strongly decom-
posed the wood of D. regia, whereas weight losses on F. benjamina
and J. minosofolia were significantly lower. By contrast, only negligi-
able weight losses were recorded from A. bidwillii. The variations
in weight loss may be explained by the specific lignin composition
of the different wood species (Schwarz, 2007). Gymnospermous
wood consists almost exclusively of guaiacyl monomers, whereas
angiospermous wood consists of approximately equal ratios of
guaiacyl and syringyl monomers (Whetten and Sederoff, 1995).
Fungal decay types fall into three categories according to their
mode of degradation of the woody cell wall: brown rot, white rot
and soft rot (Schwarz, 2007). White rot is subdivided into simul-
taneous rot and selective delignification. Ultrastructural studies
have revealed that P. noxius causes a simultaneous rot (Nicole
et al., 1995). Many basidiomycetes cause simultaneous rot in
angiosperms, but only rarely in gymnospermous wood (Schwarz,
2007). This selection may be related to the extremely resilient S3
layer of tracheids, which hampers degradation by hyphae from
within the cell lumen outwards (Schwarz, 2007), and may further
explain the resistance of gymnospermous wood to degradation by
P. noxius. The high resistance of A. bidwillii wood to decomposition by
different P. noxius strains appears to suggest an enhanced dura-
bility of softwoods to decay. However, Ann et al. (2002) report that
wood of other Araucaria species (e.g. A. cunninghamii) is susceptible
to decomposition by P. noxius.

On the basis of the present study results, the selection of one
Trichoderma strain for biological control of P. noxius is difficult.

The European strain, T. atroviride 15603.1, showed a high antago-
nistic potential against all wood-decay fungi and its VOCs greatly
inhibited their growth. However, its failure to grow at 35 °C indi-
cates its limited adaptation to the subtropical climate of Brisbane
where temperatures can exceed 35 °C. Even at high temperatures
and low a, T-4510 was a very competitive species and mycoapa-
sitism in wood resulted in a reduction in wood weight losses, but
its VOCs actually stimulated the growth of some P. noxius strains.
These results demonstrate that one and the same Trichoderma spe-
cies may possess positive and negative attributes for biological
control. As one Trichoderma species rarely has only positive attri-
butes, the selection of one potential Trichoderma species is not a
trivial exercise and will require further screening studies. For bio-
logical control a number of Trichoderma species with different
antagonistic properties may have to be combined to successfully
inhibit the growth of the target pathogen in the field.

In conclusion, the present study shows that the success of bio-
logical control of P. noxius depends on the Trichoderma species, the
strain of wood-decay fungus, the specific wood species and the
prevailing environmental conditions (temperature/water activity).
The in vitro screening of antagonistic potential used in this study
allowed a systematic investigation of several Trichoderma strains,
as well as specific ecological factors, and thus identification of
effective strains. The study shows that Trichoderma species can be
used to significantly inhibit the growth of different P. noxius
strains, and have a lethal effect on some basidiomycota species in
controlled growing conditions. The study also showed that Trich-
oderm a species can be used to reduce the rate of decomposition by
P. noxius and Ganoderma australe in the wood of the species studied
here. However, positive results obtained from in vitro studies are
only indicative, as the results of experimental conditions do not take all eco-
logical and endemic factors into account. For this reason, field studies
are essential to test the selected competitive biocontrol agent un-
der field conditions (Schubert et al., 2008b).

Acknowledgments

We thank Brisbane City Council for assisting in the preparation
of wood materials and facilities, and Keith Foster for technical and
project support. Finally, the financial and technical support (i.e.
data collection and study design) provided by ENSPEC and Brisbane
City Council is gratefully acknowledged.

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

Mark Schubert¹,², Siegfried Fink², Francis W.M.R. Schwarze¹,²

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 (HIELJORD & 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 (SCHRIMBÖCK 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 (SCHRÄRZER et al., 1999, SCHRÄRZER & 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, SCHRÄRZER et al., 1999). The ability of *Ganoderma adspersum* to degrade polyphenolic deposits in reaction zones was recently demonstrated by SCHRÄRZER & 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

<table>
<thead>
<tr>
<th><em>Trichoderma</em></th>
<th>Isolate-N°</th>
<th>Wood decay fungi</th>
<th>Isolat-N°</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichoderma atroviride</em> Karsten</td>
<td>15603.1¹</td>
<td><em>Polyporus squamosus</em> (Hud.:Fr.) Fr.</td>
<td>291101.2¹</td>
</tr>
<tr>
<td><em>Trichoderma atroviride</em> Karsten</td>
<td>CBS 351.93²</td>
<td><em>Ganoderma adspersum</em> (S. Schulz.) Donk</td>
<td>086699.2¹</td>
</tr>
<tr>
<td><em>Trichoderma atroviride</em> Karsten</td>
<td>CBS 396.92²</td>
<td><em>Ganoderma lipsiense</em> (Batsch) Atk.</td>
<td>250593.1¹</td>
</tr>
<tr>
<td><em>Trichoderma fasciculatum</em> (strictipile) Bissett*</td>
<td>CBS 338.93²</td>
<td><em>Inonotus hispidus</em> (Bull.:Fr.) Karsten</td>
<td>200792.1¹</td>
</tr>
<tr>
<td><em>Trichoderma virens</em> Miller, Giddens &amp; Foster</td>
<td>CBS 126.65²</td>
<td><em>Kretzschmaria deusta</em> (Hoffm.) P.M.D. Mar.</td>
<td>271098.1¹</td>
</tr>
<tr>
<td>BINAB TF WP (<em>T. harzianum/T. polysporum</em>)</td>
<td>IMI 206039/40³</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 then 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 \textit{Trichoderma} spp. is provided in Tables 2 and 3. Growth rates of all \textit{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 \textit{Trichoderma} isolates was greatly enhanced.

The highest temperature supporting growth was recorded on MEA at 25(±1)\(^\circ\)C and on LNA at 30(±1)\(^\circ\)C. All \textit{Trichoderma} isolates showed a growth and germination optimum at the highest water activity of \( a_w \) 0.998 and at 25(±1)\(^\circ\)C. Significant differences between the \textit{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%). 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 \textit{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 \textit{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 \textit{Trichoderma} spp. concerning the production and effect of VOCs, the wood decay fungi differed significantly in their reaction to the VOCs \( (P<0.001) \). \textit{I. hispidus} and \textit{G. adspersum} showed a strong sensitivity to the VOCs followed by \textit{P. squamosus}.
Table 2. Mean growth rate of the *Trichoderma* spp. under different conditions (mm d⁻¹). ± SE

<table>
<thead>
<tr>
<th>Temperature</th>
<th>MEA</th>
<th>LNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>aₜ 0.892</td>
<td>aₜ 0.995</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>6.4 ± 0.15</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>5.6 ± 0.23</td>
</tr>
</tbody>
</table>

Table 3. Mean germination rate of *Trichoderma* spp. under different conditions (% d⁻¹). ± SE.

<table>
<thead>
<tr>
<th>Water activity</th>
<th>MEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>aₜ</td>
<td>Temperature °C</td>
</tr>
<tr>
<td>0.892</td>
<td>5</td>
</tr>
<tr>
<td>0.995</td>
<td>0</td>
</tr>
<tr>
<td>0.998</td>
<td>0</td>
</tr>
</tbody>
</table>
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
The following table shows the classification of the degree of mycoparasitism of different *Trichoderma* spp. on MEA. ± SE.

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

| 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.

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

*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.
After 12 and 18 weeks the differences increased and were highly significant \((P<0.001)\). The additives used in conidial suspension 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* spp. 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 \((P<0.05)\).
IN VITRO SCREENING OF AN ANTAGONISTIC TRICHODERMA STRAIN

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.

<table>
<thead>
<tr>
<th></th>
<th><em>Inonotus hispidus</em></th>
<th><em>Ganoderma adspersum</em></th>
<th><em>Ganoderma lipsiense deusta</em></th>
<th><em>Kretzschmaria squamosus</em></th>
<th><em>Polyporus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 w</td>
<td>12 w</td>
<td>18 w</td>
<td>6 w</td>
<td>12 w</td>
</tr>
<tr>
<td><strong>T-15603.1</strong></td>
<td>62.28*</td>
<td>58.84*</td>
<td>61.58*</td>
<td>59.79*</td>
<td>75.77**</td>
</tr>
<tr>
<td><strong>T-351.93</strong></td>
<td>64.07*</td>
<td>60.00*</td>
<td>62.30*</td>
<td>60.82*</td>
<td>76.59**</td>
</tr>
<tr>
<td><strong>T-396.92</strong></td>
<td>22.75n.s</td>
<td>54.78*</td>
<td>19.75n.s</td>
<td>60.82*</td>
<td>60.33*</td>
</tr>
<tr>
<td><strong>T-Binab</strong></td>
<td>20.96n.s</td>
<td>53.33*</td>
<td>17.59n.s</td>
<td>60.82*</td>
<td>60.16*</td>
</tr>
<tr>
<td><strong>T-126.65</strong></td>
<td>14.37n.s</td>
<td>48.12*</td>
<td>42.73*</td>
<td>57.39*</td>
<td>69.27**</td>
</tr>
<tr>
<td><strong>T-338.93</strong></td>
<td>10.78n.s</td>
<td>34.49n.s</td>
<td>49.01*</td>
<td>49.14*</td>
<td>69.76**</td>
</tr>
</tbody>
</table>
| **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* ≥ 0.05)
Table 7. Reduction (%) of the wood decay (wood weight loss) by applying conidial suspension 2 of *Trichoderma* spp.

<table>
<thead>
<tr>
<th>Conidial Suspension 2</th>
<th>Inonotus hispidus</th>
<th>Ganoderma adspersum</th>
<th>Ganoderma lipsiense</th>
<th>Kretzschmaria deusta</th>
<th>Polyporus squamosus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 w</td>
<td>12 w</td>
<td>18 w</td>
<td>6 w</td>
<td>12 w</td>
</tr>
<tr>
<td>T-15603.1</td>
<td>63.47*</td>
<td>67.25*</td>
<td>73.25*</td>
<td>78.35*</td>
<td>86.83**</td>
</tr>
<tr>
<td>T-351.93</td>
<td>64.67*</td>
<td>68.12*</td>
<td>73.43*</td>
<td>78.35*</td>
<td>87.64**</td>
</tr>
<tr>
<td>T-396.92</td>
<td>25.15n.s</td>
<td>59.71*</td>
<td>52.42*</td>
<td>61.51*</td>
<td>61.46*</td>
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<tr>
<td>T-Binab</td>
<td>25.75n.s</td>
<td>59.13*</td>
<td>50.27*</td>
<td>61.86*</td>
<td>61.46*</td>
</tr>
<tr>
<td>T-126.65</td>
<td>43.71n.s</td>
<td>19.16*</td>
<td>53.62*</td>
<td>67.70*</td>
<td>37.46**</td>
</tr>
<tr>
<td>T-338.93</td>
<td>52.10n.s</td>
<td>59.13n.s</td>
<td>69.48*</td>
<td>68.73*</td>
<td>77.89**</td>
</tr>
</tbody>
</table>

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.
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 (KULNING 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.

**References**


Weindling, R. (1934) Studies on lethal principle effective in the parasitic action of Trichoderma lignorum on Rhizoctonia solani and other soil fungi. Phytopathology, 24, 1153–1179.


FIELD EXPERIMENTS TO EVALUATE THE APPLICATION OF TRICHODERMA STRAIN (T-15603.1) FOR BIOLOGICAL CONTROL OF WOOD DECAY FUNGI IN TREES

Mark Schubert¹,² Siegfried Fink², Francis W.M.R. Schwarze¹,²

Field experiments were carried out at different locations and on hosts with T-15603.1, a Trichoderma strain for biological control of wood decay fungi. The objective of the studies was to monitor and optimize conditions for colonization of the antagonist, its survival in time and space and to improve its effectiveness as wound treatment method. A total of 159 angiosperm trees and 1431 wounds from six different species (Platanus x hispanica, Acer pseudoplatanus, Tilia platyphyllos, Populus nigra, Quercus rubra, Robinia pseudoacacia) were treated with different conidial suspensions of T-15603.1. In comparison to untreated control wounds, T-15603.1 significantly suppressed growth (82.3%) of wounds colonised by three basidiomycetes Ganoderma adspersum, Inonotus hispidus and Polyporus squamosus (P<0.001). Monitoring results with RAPD-PCR showed that spore suspensions applied in a humidity storing gel as a carrier suspension significantly increased the germination rate and therefore colonization of the wound surface by T-15603.1 (P<0.001). Interpretation and characterization of the isolated microorganisms such as diversity and succession were analyzed using diversity indices. The results demonstrate that T-15603.1 can be successfully applied as a biological wound treatment against wood decay fungi of urban trees.

Introduction

Since the pioneering work of RISHBETH (1961), interest in the genus Trichoderma as competitive antagonists against important pathogens of forest trees such as Heterobasidion annosum (Fr.:Fr.) Bref. and Armillaria spp. has increased steadily (HOLDENRIEDER, 1984; NICOLOTTI et al., 1999; FOX, 2003). GROSCLAUDE et al. (1973) and CORKE (1980) successfully applied T. viride Pers. Ex S.F. Grey as wound treatment method for fruit trees against the wound parasite Chondrostereum purpureum (Pers. ex Fr.) Fr.

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In studies by Pottle & Shigo (1975) and Pottle et al. (1977) 
Trichoderma harzianum Rifai treatment of wounds of Acer rubrum L. with Trichoderma inhibited colonization even after 21 months.

Currently there is little knowledge on the effectiveness of biological control of wood decay fungi which colonize wounds of urban trees. On urban sites air temperature is usually higher than in forests. Moreover, soil permeability is often low due to compaction causing additional stress to the root system of trees. These factors, together with repeated pruning operations, can, alone or in combination, suppress tree health and in the presence of pathogens, trigger disease. It is also interesting to note that 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 on root plates may promote infection by wound decay fungi. *In vitro* screening is the first step for utilizing the full potential of Trichoderma species. In a previous study we identified and selected a highly competitive *Trichoderma atroviride* strain (T-15603.1) in several *in vitro* tests against five basidiomycetes *Ganoderma adspersum* (S. Schulz.) Donk, *Ganoderma lipsiense* (Batsch) Atk., *Inonotus hispidus* (Bull.:Fr.), *Polyporus squamosus* (Hud.:Fr.) Fr. and one ascomycete *Kretzschmaria deusta* (Hoffm.) P.M.D. Martin (Schubert et al., 2008). The objective of the present work was to evaluate the potential of the highly competitive strain (T-15603.1) in field experiments for a biological treatment method of pruning wounds on urban trees against colonization by wound decay fungi.

**Materials and Methods**

Field experiments were undertaken on two urban sites in Freiburg-Lehen, Baden-Württemberg (278m above sea level, 10°C; precipitation approx 1000mm per annum), Germany and Ludwigshafen, Rheinland-Pfalz (96m above sea level, 9.2°C; precipitation approx 500mm per annum), Germany. A total of 159 trees were pruned (Table 1). A total of 1431 wounds with a mean diameter of 6.4cm were treated in July and August 2003 with three different types of conidial suspensions of T-15603.1:

1. Suspension (CFU: $10^5$/ml)
2. Suspension (CFU: $10^5$/ml + 0.2% D-glucose + 0.1% urea)
3. Suspension (CFU: $10^5$/ml + 0.2% D-glucose + 0.1% urea + 0.4% sodiumpolyacrylate, a component of the product Luquasorb 1030, BASF AG, Ludwigshafen, Germany.
Conidial suspensions 1 and 2 were sprayed onto the wound surface; the third was applied with a conventional brush (Figure 1). Untreated wounds served as controls. In addition, artificial inoculation tests were performed on selected trees to evaluate the preventive effect of the *Trichoderma*-strain T-15603.1 against colonization by wood decay fungi (Table 1). Tree wounds were treated with the antagonist as described above and three weeks after treatment were inoculated with three basidiomycetes: *I. hispidus*, *G. adspersum* and *P. squamosus*. The determination of the infection rate of the latter fungi was based on inoculated wounds from which the respective fungus could be re-isolated (GADGIL & BAWDEN, 1982). The biological treatment efficacy \( E \) of T-15603.1 was measured according the formula of ABBOTT (1925):

\[
E = \frac{(Ix[\%] - IT[\%]) \times 100}{Ix[\%]}
\]

\( Ix \) = Infection rate without treatment and \( IT \) = Infection rate after application of T-15603.1.

**Re-isolation and analysis**

Establishment in the wood substrate and the persistence of T-15603.1 were monitored with standardized re-isolations after 2, 8, 12, 18, 24 and 30 months. Wood samples (20 × 10 × 5mm) were extracted from the centre and peripheral regions of the wound with a sterile chisel. In the laboratory, the surface of the samples were sterilized with hydrogen peroxide, divided into 3 subsamples and placed onto Petri dishes containing malt extract agar (MEA), TSM and T-MEA. After thirty months sections of the treated wounds were extracted, bisected radially and re-isolations were performed along a gradient and in wood to a depth of 1, 3 and 5cm. Parameters like

<table>
<thead>
<tr>
<th>Tree species</th>
<th>Location</th>
<th>Quantity</th>
<th>Inoculation</th>
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</thead>
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<tr>
<td><em>Platanus x hispanica</em> Münchh.</td>
<td>Ludwigshafen</td>
<td>54</td>
<td>–</td>
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<tr>
<td><em>Acer pseudoplatanus</em> L.</td>
<td>Ludwigshafen/Freiburg-Lehen</td>
<td>40</td>
<td>P1,P2,P3</td>
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<tr>
<td><em>Tilia platyphyllos</em> Scop.</td>
<td>Freiburg-Lehen</td>
<td>24</td>
<td>P1,P2,P3</td>
</tr>
<tr>
<td><em>Populus nigra</em> L.</td>
<td>Ludwigshafen / Freiburg-Lehen</td>
<td>16</td>
<td>P1,P2,P3</td>
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<tr>
<td><em>Quercus rubra</em> L.</td>
<td>Freiburg-Lehen</td>
<td>16</td>
<td>P1,P2,P3</td>
</tr>
<tr>
<td><em>Robinia pseudoacacia</em> L.</td>
<td>Ludwigshafen</td>
<td>9</td>
<td>–</td>
</tr>
</tbody>
</table>

P1 = *Inonotus hispidus*
P2 = *Ganoderma adspersum*
P3 = *Polyporus squamosus*
wound occlusion and discoloration were recorded and measured according to LIese et al. (1988) and Metzler (1997) (Figure 2 A&B). Additional data such as site, orientation, climate data, ratio of sap- and heartwood and wound dimensions were also recorded periodically.

A statistical interpretation and characterization of the isolated fungal populations, i.e. diversity and succession, was made using diversity indices (Maria & Sridhar, 2002).

Shannon Index $H'$:

$$H' = - \sum_{i=1}^{S} p_i \cdot \log(p_i)$$

$S$ = number of different species or groups; $N$ = total numbers of individuals; $n_i$ = number of individuals classified as $i$th species or group; $p_i$ = proportion of total samples classified as $i$th species.

The Shannon Equitability $E$ is the ratio between the observed species diversity ($H'$) and the maximum species diversity ($H_{\text{max}}$) calculated as:

$$E = \frac{H'}{H_{\text{max}}} + \frac{H'}{\log(S)}$$

$E$ = equitability (range 0-1, max. value when all species are equally abundant); $H$ = observed species diversity; $H_{\text{max}}$ = species diversity under conditions of maximal evenness.
Identification of the obtained isolates was undertaken both with traditional 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, relying on the microscopic characteristics of conidiophores. Identity was established using several diagnostic keys (RIFAI, 1969; BISSETT, 1984, 1991a,b,c, 1992; GAMS & BISSETT, 1998). For DNA isolation, fungal cultures were grown at room temperature on 2% MEA. Mycelia were harvested by filtration through a piece of filter paper and washed with distilled water. The samples were immediately frozen in liquid nitrogen and lyophilized.

DNA extraction was 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 260nm (Beckmann DU 7500i, Munich). RAPD characters were developed with primer 1 (5’-CACGCGAGT-3’) and primer 2 (5’-CTGTCGAGCA-3’) (Carl Roth GmbH). The reaction volume of 50 μl contained 26.5 μl distilled water, 5μl Mg-reaction buffer, 5μl 25mM MgCl2, 5μl Primer 1 and 2, 1μl 10mM dNTP mix, 0.5μl Taq DNA polymerase, 2μl DNA. 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) and with a final extension step for 10 min. at 72°C. The DNA samples were separated for analysis by electrophoresis on 1 to 2% agarose gels (1× Tris-borate EDTA buffer). The fragments were visualized by staining with 0.5μg of ethidium bromide per ml and UV illumination.

Results

Monitoring results revealed successful re-isolation of the *Trichoderma* strain T-15603.1 from treated wounds thirty months after application (Figure 3). The establishment and colonization of T-15603.1 on the wound surface was highly dependent on the medium in which the conidia were suspended (Table 2). Table 3 shows contrast analysis of the different conidial suspensions tested. The highest mean re-isolation rate of 74.8% was recorded after application with conidial suspension 3 (P<0.05). Wounds were more weakly colonized after treatment with suspensions 1 (32.5%) and 2 (29.1%). Re-isolation rates from wounds treated with suspension 3 were significantly higher than from control wounds (P<0.001). After twelve months no differences between wound treatment with suspension 2 and control wounds were found. After thirty months no differences were observed between the re-isolation rates of suspension 1 and the controls. Correlation

![Figure 3. RAPD-PCR: (1) = The applied strain T-15603.1 (reference). (2-6) = *Trichoderma*-isolates from the treated pruning wounds after 30 months. (7) = *Trichoderma virens*. (8) = *Trichoderma fasciculatum*.](image-url)
FIELD EXPERIMENTS TO EVALUATE THE APPLICATION OF TRICHODERMA STRAIN

Table 2. Re-isolation rate of T-15603.1 (%) from pruning wounds in relation to different conidial suspensions applied.

<table>
<thead>
<tr>
<th>Treatment method of conidial suspensions</th>
<th>Time [months]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Suspension 1&lt;sup&gt;a&lt;/sup&gt; vs. Control</td>
<td>0.038</td>
</tr>
<tr>
<td>Suspension 2&lt;sup&gt;b&lt;/sup&gt; vs. Control</td>
<td>0.001</td>
</tr>
<tr>
<td>Suspension 3&lt;sup&gt;c&lt;/sup&gt; vs. Control</td>
<td>0.001</td>
</tr>
<tr>
<td>Suspension 2&lt;sup&gt;b&lt;/sup&gt; vs. Suspension 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.055</td>
</tr>
<tr>
<td>Suspension 3&lt;sup&gt;c&lt;/sup&gt; vs. Suspension 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.000</td>
</tr>
<tr>
<td>Suspension 3&lt;sup&gt;c&lt;/sup&gt; vs. Suspension 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.014</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Conidial suspension (CFU 10<sup>5</sup> ml<sup>-1</sup>) without additives
<sup>b</sup> = Conidial suspension (CFU 10<sup>5</sup> ml<sup>-1</sup>) with 0.1% urea; 0.2% glucose
<sup>c</sup> = Conidial suspension (CFU 10<sup>5</sup> ml<sup>-1</sup>) with 0.1% urea; 0.2% glucose and 0.4% sodiumpoly-acrylate

F-test of significance P<0.05, P value is denoted, not significant at P ≥ 0.05

Table 3. Contrast analysis of the re-isolation rate of T-15603.1 from pruning wounds in relation to the different conidial suspensions applied.

<table>
<thead>
<tr>
<th>Treatment method of conidial suspensions</th>
<th>2</th>
<th>8</th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspension 1&lt;sup&gt;a&lt;/sup&gt; vs. Control</td>
<td>0.038</td>
<td>0.001</td>
<td>0.042</td>
<td>0.045</td>
<td>0.048</td>
<td>0.056</td>
</tr>
<tr>
<td>Suspension 2&lt;sup&gt;b&lt;/sup&gt; vs. Control</td>
<td>0.001</td>
<td>0.046</td>
<td>0.052</td>
<td>0.058</td>
<td>0.078</td>
<td>0.167</td>
</tr>
<tr>
<td>Suspension 3&lt;sup&gt;c&lt;/sup&gt; vs. Control</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Suspension 2&lt;sup&gt;b&lt;/sup&gt; vs. Suspension 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.055</td>
<td>0.061</td>
<td>0.089</td>
<td>0.092</td>
<td>0.104</td>
<td>0.096</td>
</tr>
<tr>
<td>Suspension 3&lt;sup&gt;c&lt;/sup&gt; vs. Suspension 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.000</td>
<td>0.019</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Suspension 3&lt;sup&gt;c&lt;/sup&gt; vs. Suspension 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.014</td>
<td>0.000</td>
<td>0.001</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Conidial suspension (CFU 10<sup>5</sup> ml<sup>-1</sup>) without additives
<sup>b</sup> = Conidial suspension (CFU 10<sup>5</sup> ml<sup>-1</sup>) with 0.1% urea; 0.2% glucose
<sup>c</sup> = Conidial suspension (CFU 10<sup>5</sup> ml<sup>-1</sup>) with 0.1% urea; 0.2% glucose and 0.4% sodiumpoly-acrylate

F-test of significance P<0.05, P value is denoted, not significant at P ≥ 0.05

Analysis according to Spearman’s rho showed a positive relationship between precipitation and re-isolation rate (<rho>=0.829, P<0.05) and a negative correlation between wound dimension and re-isolation rate (<rho>=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 significant correlation between re-isolation rate and temperature was detected (<rho>=0.371, P<0.05), nor between re-isolation rate and tree species (<rho>=0.276, P<0.05).

Thus the applied conidial suspensions, the moisture content of the wood and the ratio of sap- and heartwood significantly (P<0.05) influenced the re-isolation rate of T-15603.1 (Figure 4). T-15603.1 was re-isolated from the sapwood (69.7%) more often than from the heartwood (30.4%). After two months the re-isolation rate of T-15603.1 was similar from the sap- and
heartwood, but after eight months a significant difference between sap- and heartwood was detectable (P<0.05). This trend persisted and after eighteen months the differences between re-isolation rates from sap- and heartwood were highly significant (P<0.001). Isolations from different wood depths showed that the highest re-isolation rate was obtained from the surface of the wounds (46.1%). Re-isolation of T-15603.1 decreased significantly (P<0.05) in deeper wood regions and at a depth of 5cm T-15603.1 was not detectable (Table 4).

Analysis of wood discolouration resulting from pruning wounds showed that the maximum amount of wood discolouration was detected in *Populus nigra* (20.4cm²), followed by *Tilia platyphyllos* (16.74 cm²) and *Acer pseudoplatanus* (11.8cm²). The smallest amount of wood discolouration was observed in *Quercus rubra* (9.5cm²). In addition a positive correlation (<rho>=0.721) was observed for wood discolouration and wound dimension. Moreover the treatment method had a considerable effect on the expansion

<table>
<thead>
<tr>
<th>Table 4. Re-isolation rate of T-15603.1 in different wood depths (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspension 1</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>1 cm</td>
</tr>
<tr>
<td>3 cm</td>
</tr>
<tr>
<td>5 cm</td>
</tr>
</tbody>
</table>

Letters denote significant differences after contrasts analysis P < 0.05

**Figure 4.** Re-isolation rate of T-15603.1 from the sap- and heartwood (%) of wounds. Asterixes indicate significant differences *=significant (P<0.05); **=highly significant (P<0.001).
of wood discolouration. Development of discoloured wood was more extensive from wounds inoculated with the wood decay fungi and without pre-treatment than wounds treated with T-15603.1 (Figure 5). Thus pre-treatment significantly (P<0.05) reduced development of dysfunctional wood. No differences in wood discolouration were recorded for the different wound decay fungi (P<0.05).

After thirty months the woundwood coefficient (wound occlusion) was measured and analysed. A mean woundwood coefficient of 71.3% was determined (c_{coef} 71.3% of the wound was occluded). Figure 6 provides statistical analysis of the woundwood development of different tree species. The highest woundwood coefficient was measured for Populus nigra (c_{coef} 79.6%), followed by Tilia platyphyllos (c_{coef} 72.7%), Acer pseudoplatanus (c_{coef} 69.2%) and Quercus rubra (c_{coef} 63.9%). By contrast no significant differences were observed between the tree species Tilia platyphyllos, Acer pseudoplatanus and Quercus rubra; the coefficients for Populus nigra and Acer pseudoplatanus, and for Quercus rubra, were significantly different (P<0.05).

The effect of treatment with T-15603.1 on the inoculated wood decay fungi and wound occlusion is apparent from Figure 7. The woundwood coefficients were divided into 3 categories (1=c_{coef} <30%, 2=c_{coef} 31–60%, 3=c_{coef} >60%). Wounds treated with T-15603.1 and untreated controls could be classified in the highest category 3 (>60% wound closure), whereas wounds inoculated with wood decay fungi could be classified in category

![Figure 5. Extent of wood discolouration developing from pruning wounds. Letters denote significant differences after contrasts analysis P<0.05](image-url)
Figure 6. Measurement of the woundwood coefficient. Letters denote significant differences (P<0.05) of wound occlusion for tree species.

![Diagram](image)

\[ C\% = 100 \times \frac{(\pi \times h \times b)}{\pi \times r^2} \times 100 \]

(1) <30%  (2) 31–60%  (3) >60%

T-15603.1  
Wood decay fungi  
Control

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Category 1</th>
<th>Category 2</th>
<th>Category 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-15603.1</td>
<td>10 [19.2%]</td>
<td>15 [28.8%]</td>
<td>27 [51.9%]</td>
</tr>
<tr>
<td>Wood decay fungi</td>
<td>15 [26.3%]</td>
<td>26 [45.61%]</td>
<td>22 [38.6%]</td>
</tr>
<tr>
<td>Control</td>
<td>4 [18.2%]</td>
<td>5 [22.7%]</td>
<td>13 [59.1%]</td>
</tr>
</tbody>
</table>

Figure 7. Wound occlusion rate (woundwood coefficient) for individual treatments was classified in 3 woundwood coefficient categories (1=\(c_{\text{coef}}<30\%\), 2=\(c_{\text{coef}} 31-60\%\), 3=\(c_{\text{coef}} >60\%\)).

2 (31–60% wound occlusion). The results indicated that the wood decay fungi negatively influenced wound occlusion, whereas neither a positive nor negative effect on woundwood formation was recorded for T-15603.1. In addition to tree species and wound treatment, wound size influenced wound occlusion. In Figure 8 the influence of the wound size on the woundwood coefficient is illustrated. According to Spearman’s rho analysis a negative correlation (\(<\rho>=0.6879\) was recorded for woundwood coefficient and wound size (P<0.05). Thus with increasing wound size the woundwood coefficient decreased.
In addition to bacteria, a range of fungal genera and species were isolated from the untreated wounds (Table 5). The application of T-15603.1 reduced the microbial diversity of colonized wounds (Table 6). Thus, the mean indices of the untreated wounds (control) $H'=1.547$ and $E=0.846$ were higher than the indices of the treated wounds ($H'=0.829$ and $E=0.414$). Wounds treated with conidial 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 a high biocontrol efficacy of T-15603.1 against *I. hispidus*, *G. adspersum* and *P. squamosus* on pruning wounds (Figure 9). 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 similar statistical performance ($P<0.05$). Analysis of variance showed that the treatment of pruning wounds with conidial suspensions of T-15603.1 reduced 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 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%; $P<0.05$)

![Figure 8. Correlation analysis showed a significant relationship between wound size and woundwood coefficient ($P<0.05$).](image-url)
TABLE 5. Fungal genera and species isolated from untreated pruning wounds.

<table>
<thead>
<tr>
<th>Deuteromycetes</th>
<th>Ascomycetes</th>
<th>Zygomycetes</th>
<th>Basidiomycetes</th>
<th>Myxomycetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acremonium sp.</td>
<td>Pestalotia sp.</td>
<td>Aleuria sp.</td>
<td>Absidia sp.</td>
<td>Physarum sp.</td>
</tr>
<tr>
<td>Alternaria sp.</td>
<td>Penicillium sp.</td>
<td>Apiognomonia sp.</td>
<td>Mortierella sp.</td>
<td></td>
</tr>
<tr>
<td>Mortierella sp.</td>
<td>Phialophora sp.</td>
<td>Ascocoryne sp.</td>
<td>Stereum hirsutum</td>
<td></td>
</tr>
<tr>
<td>Aureobasidium sp.</td>
<td>Phoma sp.</td>
<td>Chaetomium sp.</td>
<td>Rhizopus sp.</td>
<td></td>
</tr>
<tr>
<td>Cladosporium sp.</td>
<td>Nodulisporium sp.</td>
<td>Daldinia sp.</td>
<td>Flammulina velutipes</td>
<td></td>
</tr>
<tr>
<td>Curvularia sp.</td>
<td>Stemphylium sp.</td>
<td>Erysipha sp.</td>
<td>Stereum hirsutum</td>
<td></td>
</tr>
<tr>
<td>Epicoccum sp.</td>
<td></td>
<td>Hypocrea sp.</td>
<td>Trametes versicolor</td>
<td></td>
</tr>
<tr>
<td>Exophiala sp.</td>
<td></td>
<td>Nectria sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusarium sp.</td>
<td></td>
<td>Peziza sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gliocladium sp.</td>
<td></td>
<td>Verticillium sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocillium sp.</td>
<td></td>
<td>Xylaria sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paecilomyces sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 6. Influence of T-15603.1 application on microbial diversity

<table>
<thead>
<tr>
<th>Diversity indices</th>
<th>Suspension 1</th>
<th>Suspension 2</th>
<th>Suspension 3</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>H'</td>
<td>0.811</td>
<td>1.024</td>
<td>0.651</td>
<td>1.547</td>
</tr>
<tr>
<td>E</td>
<td>0.468</td>
<td>0.534</td>
<td>0.242</td>
<td>0.846</td>
</tr>
</tbody>
</table>

Figure 9. Reduction of the infection rate of wood decay fungi on pruning wounds after treatment with T-15603.1. Asterices indicate significant differences (F-test) for untreated controls and treated wounds (* = P<0.05; ** = P<0.001).

Discussion

The field screening trials were undertaken to evaluate the potential of the selected *T. atroviride* isolate 15603.1 for biological control of wood decay fungi under natural conditions. Optimal establishment of the antagonist in the wood substrate prior to colonization by a wound pathogen appears to be a good strategy for a successful wound protection treatment. Establishment is highly dependent on the adhesion and the viability of 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 the performance of biocontrol agents is by formulating conidial suspensions that enhance the adhesion and viability. In this study a range of different conidial suspensions of T-15603.1 was formulated and tested, which significantly...
Figure 10. A: Completely occluded wound of *Populus* sp. B: Occluded poplar wound pre-treated with *Trichoderma* sp. (longitudinal section). C: Longitudinal section of pruning wound of *Tilia* sp. Discoloured wood showed a bleached appearance [B]. RZ = reaction zone. D: Pre-treated wound surface of *Quercus* sp. showing no signs of degradation. E: Visible degradation of wound surface (*Tilia* sp.) inoculated with *G. adspersum* without pre-treatment. F + G: Zone lines indicate successful colonization of wounds by *P. squamosus*. H: Wood of *Tilia* wounds inoculated with *I. hispidus* was strongly degraded after 3 years [D]. RZ = reaction zone. I: Untreated wound (control) with fruit bodies of *Stereum hirsutum* (Willd.) Pers.
influenced the establishment (P<0.001) of the antagonist in pruning wounds. 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 conidal germination. Conidial suspension 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 demonstrated the existence of a significant relationship between an increase in germination rate, viability and biocontrol efficacy of Trichoderma spp. However the authors also mentioned that conidia that are enriched in nutrients showed an increased water requirement resulting in a reduced germination rate due to a reduction in 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 possibly limited due to an increased water requirement and due to desiccation of the wood substrate i.e. the establishment, as indicated by re-isolation rate of T-15603.1, was hampered. Furthermore, the effect of the additives was not limited to the Trichoderma isolate but may also have promoted microflora competition in the wood substrate as indicated by the higher diversity indices from wounds treated with conidial suspension 2 than from wounds treated with suspension 1. In previous studies Simon & Sivathamparam (1988) observed the inhibition of Trichoderma spp. by different bacteria in dual culture tests. Naár & Kecskés (1998) demonstrated that in several in vitro tests the bacteria Clavibacter michiganese and Pseudomonas syringae caused a significant inhibition of Trichoderma spp. by the production and excretion of antibiotic substances. Lutz et al. (2003) observed a negative influence of the chitinase gene expression and therefore a reduction of antagonistic activity by the fungi Fusarium culmorum and F. graminearum.

Carriers of inocula are inert substances in the sense that they do not have a disease control capacity; however they can profoundly affect time of germination as well as viability of conidia (Favel et al., 1998). In the present study, the conidia of T-15603.1 enriched by additives in a humidity-storing gel formulation provided a constant moisture reservoir and thus increased conidial viability and effectiveness, as indicated by the significantly (P<0.001) higher re-isolation rates from wounds treated with the conidial suspension 3. In addition, conidial adhesion to the wood substrate was improved by the use of the gel as carrier substance (Batta, 2004; Jayaraj et al., 2006). Both increased viability and adhesion enhanced the establishment of T-15603.1 and thus improved efficiency as indicated by the lowest diversity indices recorded from wounds inoculated with the conidial suspension 3. In particular the low E index 0.242 (high ratio of
the antagonist on total number of individuals or groups) demonstrates that T-15603.1 was the dominant species in the wood substrate.

The isolation rate of naturally occurring *Trichoderma* spp. from the untreated control wounds was consistently very low (4.5%). There is no explanation for this at present other than the speculation that the presence of *Trichoderma* spp. on urban sites may be limited due to the absence of organic matter, which is essential for growth, survival and sporulation of the fungus. More detailed studies are needed to confirm this hypothesis. The highest diversity indices were detected from control wounds. In particular the low E index (0.846) is indicative of the fact that on untreated wounds a natural succession of microorganisms occurred in the absence of T-15603.1.

Not only the type of conidial suspensions and abiotic factors, especially wood moisture, influenced the establishment of the biocontrol agent, but also the wood substrate appeared to be a decisive factor. The re-isolation rate was significantly (P<0.05) reduced in the heartwood. *SHIGO & HILLS (1973)* investigated the specific characteristics of the heartwood from several tree species and they described heartwood as a substrate with low wood moisture content and with fungistatic properties, which hampers colonization by microorganisms. Thus, the conidial viability and consequently the biocontrol efficacy of T-15603.1 is reduced on large wounds with a high ratio of heart-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 & 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 *G. adspersum* to degrade polyphenolic deposits in reaction zones was demonstrated by *SCHWARZE & FERNER (2003)* and explains the high infection rate (78.7%) of the untreated wounds after thirty months. Pretreatment of the wounds with T-15603.1 resulted in a strong preventive effect against colonisation by wood decay fungi, particularly *I. hispidus* and *G. adspersum* (Figure 9). This observation is in good agreement with the results previously obtained in laboratory tests that showed a high susceptibility of both basidiomycetes to antagonism by T-15603.1 (*SCHUBERT et al., 2008*). A lower effect (62.4%) was measured for *P. squamosus*, which is also in good agreement with results obtained in the *in vitro* studies.

The active mechanisms of antagonism in the wood substrate are determined by rapid growth, mycoparasitism and antibiosis. A further passive effect of T-15603.1 could be related in maintaining the tree’s defence 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 to fungistatic substances, may reduce the efficiency of defence boundaries by metabolizing large amounts of polyphenols (Smith *et al.*, 1981). Inhibition and exclusion of such fungi may maintain high concentrations of polyphenols thus preventing colonization by decay fungi which are sensitive to fungistatic substances. Whether the treatment with T-15603.1 helped maintaining tree defence boundaries could not definitely be demonstrated, but the present work strongly indicates that the inoculation of T-15603.1 not only inhibits colonization by wood decay fungi but also of many other microorganisms as demonstrated by the diversity indices.

Results obtained from *in vitro* studies are helpful to eliminate non-performing isolates from the screening tests but are not representative, as *in vitro* assays do not completely mimic all ecological and endemic factors. Factors such as wood moisture content and competition with the indigenous microflora are impossible to reproduce in the laboratory. For this reason field studies are essential to test the efficacy of selected biocontrol agent under natural conditions. Results of field studies demonstrate that the *Trichoderma* strain T-15603.1 can be successful when applied as a biological wound treatment method against *I. hispidus* and *G. adspersum* on urban sites. But there is still a need to optimize the formulation of conidia i.e. to improve the establishment and therefore the biocontrol efficacy, particularly on pruning wounds with a high heart- to sapwood ratio. 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 can not completely inhibit colonization by all wood decay fungi. In addition genotype and virulence of wood decay fungi may vary from those used in this study. A strategy to enhance the effect of a biological wound treatment could be based on 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 & Roberts, 2002).

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References


FIELD EXPERIMENTS TO EVALUATE THE APPLICATION OF TRICHODERMA STRAIN


