Identification and monitoring of *Trichoderma* in field soil

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Overview

1. Introduction
2. Molecular identification of *Trichoderma* strain potentially useful as biological control agents
3. Development of PCR-based markers useful for *Trichoderma* identification and monitoring in soil
4. Monitoring of *T. atroviride* and *T. harzianum* applied in lettuce cultivation
5. Conclusions
Introduction - Agriculture in Poland – brief overview

3 in EU - agricultural area, next to France/Spain
about 25% of population employed in agriculture
3% of GDP gross domestic product
8,5ha – average farm size

Major crops, based on production area

Cereals: wheat, triticale, rye, maize
Other: potato, sugarbeet

Fruits:
apples, plums, cherries, strawberries, raspberries

Vegetables:
onion, cabbage, carrot, cucumber

Apples: world top 3 producer biggest European producer

Expatspoland.com
Tygodnik-rolniczy.pl
The importance of *Trichoderma* in agriculture

**Common fungi** in the environment with **complex taxonomy** >250 species

**Many research** on *Trichoderma* – belong to the most extensively studied fungi

**Successful stories** - *Trichoderma*-based biotechnologies

- production of enzymes for chemical/food/feed industries
- in agriculture:
  - Biological Control Agents – **BCA**
    - to control diseases/pests in environment-friendly way
  - Plant Growth Promoting Fungi – **PGPF**
    - to promote crops growth/yield

However, certain *Trichoderma species /strain* can also be pathogens
Beneficial effects of *Trichoderma* in agriculture

*Trichoderma* can help in plant production in different ways

- soil properties improvement - intensification of decomposition of organic matter
- antagonistic activity against soil microorganisms
  - competition for nutrients and space
  - environment modification - acidification, siderophores (peptides with affinity to Fe$^{+3}$)
  - secretion of antibiotics –antibiosis
  - production of lytic enzymes – mycoparasitism

In the presence of *Trichoderma* plants

- can show better growth
- develop larger surface of the root system
- acquire resistance to pathogens
  - SAR – systemic acquired resistance/ ISR – induced systemic resistance
Selection of *Trichoderma* strains for the use in biological plant protection

- Many efforts to select **efficient biocontrol strains**
- To commercialize biocontrol strain, it is **extremely important** to establish taxonomic position and to develop techniques for strain identification
- **Molecular methods** are now replacing classical fungi identification methods
- Biopreparations has to be **effective in the environment where is applied**
- *Trichoderma* population has to be maintained at satisfactory level during crop production cycle – **monitoring methods are needed**
Molecular identification of *Trichoderma* strains potentially useful in biological plant protection in Poland
Base collection of *Trichoderma* strains

- **104 TRS strains** potentially useful as BCA / PGPF
  - originated from: soils, composts, forests,
  - collected by Szczech and others at Horticulture Institute Skierniewice
  - pre-characterised as belonging to *T.atroviride, T.harzianum, T.virens*
  - part of the collection not characterized

- species reference strain

**Goal:** Molecular identification of the strains
Molecular identification methodology

DNA isolation → cells → Method optimisation

Molecular work → MLST → Rep-PCR

Bioinformatic analysis of MLST DNA sequences

Species identification

- TrichOKEY
- TrichoMARK
- TrichoBLAST

ISTH

http://www.isth.info

Bayesian phylogenetic analyses

ISTH – International Subcomission on *Trichoderma* and *Hypocrea* Taxonomy
Optimisation of DNA isolation from medium-grown *Trichoderma*

I. Different methods of fungi cultivation for DNA isolation: solid media, liquid / rotary shaken

II. Comparition of 6 different methods of DNA isolation from *Trichoderma* cells:

1. **boiling cells**, 12 min, 105 °C
5. NucleoSpin Plant II kit, *Macherey-Nagel*, Germany
6. DNeasy Plant Mini Kit, *Qiagen*, Germany

**Green frames** – best methods
## MLST - Multilocus Sequence Typing

Testing **tens of primer** combinations - 4 pairs were selected for PCR/sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Variable region</th>
<th>Gene full name</th>
<th>Primers</th>
<th>Lenght [bp]</th>
</tr>
</thead>
</table>
| *rDNA* | ITS1 | rRNA internal transcribed spacers | ITS4 / ITS6  
White et al. 1990  
Cooke and Duncan, 1997 | 620 |
|     | ITS2 | | | |
| *tef1α* | tef_int4 (large) | translation elongation factor 1-alpha | EF1_728F / TEF_LLErev  
Carbone and Kohn 1999  
Jaklish et al. 2006 | 1.260 |
|     | tef1_int5 (short) | | | |
|     | tef1_exon6 (large) | | | |
| *chi18-5* | large exon *chi*18-5 | chitinase 42 kDa | chit42_1 af / chit42_2 ar  
Kulling-Gradinger et al. 2002 | 800 |
| *rpb2* | exon *rpb2* | RNA polymerase II | RPB2_210 up  
RPB2_1450 low  
Graefenhan, unpublished | 1.200 |

**Total length of sequenced DNA** 3.880

4 non-related loci – phylogenetic markers - used for taxonomic identification
DNA sequence variability of taxonomic markers

Graphical overview of genetic variability in 4 sequenced regions for *T. atroviride* and *T. harzianum*

Oskiera et al. unpublished
### Species identification

<table>
<thead>
<tr>
<th>Region</th>
<th>Number of strains</th>
<th>Identified strain species</th>
<th>%</th>
<th>Arising issues</th>
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<tbody>
<tr>
<td>ITS1 i 2 (TrichOKEY)</td>
<td>104</td>
<td>94</td>
<td>90,3</td>
<td>Lack of reference sequences T. gamsii; T. crassum</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>Identical references</strong> T. cerinum i T. tomentosum</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>New variants of ITS</strong> T.atroviride, T.velutinum, T.cerinum</td>
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<tr>
<td>tef1</td>
<td>104</td>
<td>100</td>
<td>96,1</td>
<td>Lack of reference sequences T. gamsii, T. crassum, T. spirale, T. pleuroticola</td>
</tr>
<tr>
<td>chi18-5</td>
<td>104</td>
<td>94</td>
<td>90,3</td>
<td></td>
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<tr>
<td>rpb2</td>
<td>104</td>
<td>100</td>
<td>96,1</td>
<td></td>
</tr>
</tbody>
</table>

Species identification was successful for 100 out of 104 strains

*tef1* and *rpb2* more informative, *tef1* the most useful

## Species identification

<table>
<thead>
<tr>
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<td><em>T. viridecens</em></td>
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<td><em>T. cf. harzianum</em></td>
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<td><em>T. atrobrunneum</em></td>
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<td>Lone lineages</td>
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TRS4, TRS29 and TRS33 possibly belong to the not yet described *Trichodema* species

Bayesian phylogenetic tree
4th intron of *tef1* *Trichoderma* Rogersonii clade

**Trichoderma atroviride** phylogeny

Bayesian phylogenetic tree

4th and 5th intron tef1

*Trichoderma atroviride*

TRS18 belongs to not described so far *T. atroviride* clade

Genetic fingerprinting - rep-PCR repetitive sequence–based PCR

ERIC / REP / BOX primers for tandemly repeated DNA

Strains were grouped by their genetic similarity

Fast method for preliminary assessment of the strains

Information about genetic diversity within species

Compatibility of the method with the results of sequence analyses

Oskiera et al. unpublished
Conclusions

- **4 phylogenic loci** allowed for identification and phylogenetic placing of *Trichoderma* species – *tef1* and *rpb2* are the most informative, *tef1* is the most useful.
- There are **limitations** of taxonomic sequences databases. Phylogenetic analysis is helpful to determine taxonomic position of poorly characterized *Trichoderma* strains.
- **rep-PCR** fingerprinting is useful in preliminary *Trichoderma* identification and diversity studies.
Development of molecular markers based on PCR for identification of selected *Trichoderma* strains
Development of molecular markers for *Trichoderma* identification and monitoring

- DNA sequences of *Trichoderma*
  - own, GenBank, ISTH, SCAR, JGI
- Sequence Alignments
- Variable regions identification

Development of PCR primers

- PCR optimisation
- Species specificity verification

Development of multiplex-PCR

Identification

Monitoring

Oskiera et al. (2017) Biological Control 113: 65-72
Species-specific PCR primers

On the basis of the tef1 and chi18-5 genes

*T. atroviride*
*T. harzianum* sensu stricto
*T. simmonsii*

On the basis of our SCAR markers developed for *T. atroviride* (Skoneczny et al. 2015)

*T. atroviride*
*T. atroviride* klad A
*T. harzianum* sensu stricto
*T. harzianum* sensu stricto, *T. simmonsii, T. atrobrunneum*
*T. harzianum* sensu stricto, *T. atrobrunneum, T. afroharzianum*
*T. harzianum* sensu stricto, *T. atrobrunneum, T. afroharzianum, T. lentiforme*

Specificity confirmed with strains representing 20 *Trichoderma* clades

Oskiera et al. (2017) Biological Control 113: 65-72
Development of diagnostic multiplex-PCR

Testing of PCR reactions with multiple sets of primers (2-4)

1. **PCR control:**
   primers specific to the fungal ITS region – 1st positive control

2. **Primers specific for the *Trichoderma* genus** 2nd positive control (*Trichoderma*)
   primers for fungal chitinase *chi18-5* or beta-tubulin *tub* gene

3. ***Trichoderma* species-specific primers** diagnostic primers
   PCR product confirms presence of specific *Trichoderma* species
   *chi18-5*, *tef1*, SCARs *T. atroviride*, QTh SCAR *T. harzianum*

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Oskiera et al. (2017) Biological Control 113: 65-72
Multiplex-PCR for *Trichoderma atroviride*

- **T. atroviride**
  - tef1
  - ITS

- **T. atroviride**
  - ITS
  - chi18-5

- **T. atroviride**
  - ITS
  - SCAR X18

- **T. atroviride klad A**
  - ITS
  - SCAR X18

Oskiera et al. (2017) Biological Control 113: 65-72
Trichoderma harzianum identification methods

Oskiera et al. (2017) Biological Control 113: 65-72
Conclusion

- DNA sequence mining allowed to develop multiplex-PCR methodology useful for *T. atroviride* and *T. harzianum* or groups of species identification
Trichoderma monitoring in mineral soil during lettuce cultivation

Control – no *Trichoderma* application
Bioprep I *T. atroviride* TRS25 + *T. harzianum* TRS59
Bioprep II *T. atroviride* TRS43 + *T. harzianum* TRS85
Organic carrier - T-GRAN - only
3 random plots for each treatment
# Field experiments in 3 locations and soil sampling

## I Experiment (spring 2012- summer 2014)

<table>
<thead>
<tr>
<th>Date</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.05.2012</td>
<td>„0” Term - before application</td>
</tr>
<tr>
<td>26.06.2012</td>
<td><em>Trichoderma</em> application</td>
</tr>
<tr>
<td>14 i 16.08.2012</td>
<td>plant harvest</td>
</tr>
<tr>
<td>11.10.2012</td>
<td>„9” Term (15 weeks since application)</td>
</tr>
<tr>
<td>9.05.2013</td>
<td>„10” Term (after winter)</td>
</tr>
<tr>
<td>10.07.2014</td>
<td>“11” Term - after more than 2 years since <em>Trichoderma</em> application</td>
</tr>
</tbody>
</table>

### Long time experiment

*Trichoderma* monitored for 2 years after application

## II Experiment (spring 2013)

<table>
<thead>
<tr>
<th>Date</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.04.2013</td>
<td>„0” Term – before application</td>
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<tr>
<td>26.04.2013</td>
<td><em>Trichoderma</em> application</td>
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<tr>
<td>15.05.2013</td>
<td>lettuce planting</td>
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<tr>
<td>13.06.2013</td>
<td>&quot;1&quot; Term - during plant growth</td>
</tr>
<tr>
<td>10.07.2013</td>
<td>plant harvest</td>
</tr>
<tr>
<td>24.07.2013</td>
<td>&quot;2&quot; Term – after plant harvesting 12 weeks since <em>Trichoderma</em> application</td>
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### Rainy weather

## III Experiment (summer 2013)

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<tr>
<th>Date</th>
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<tr>
<td>24.07.2013</td>
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<tr>
<td>24.07.2013</td>
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<tr>
<td>31.07.2013</td>
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<tr>
<td>05.09.2013</td>
<td>&quot;1” Term - during plant growth</td>
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<td>27.09.2013; 30.10.2013</td>
<td>plant harvest</td>
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<tr>
<td>03.10.2013</td>
<td>&quot;2” Term – after plant harvesting, 10 weeks since <em>Trichoderma</em> application</td>
</tr>
</tbody>
</table>

### Good plant growth and weather condition

Oskiera et al. (2017) Biological Control 113: 65-72
Monitoring of *Trichoderma* in soil

- Soil samples
- Microbiological analysis
  - Soil samples plating on Martin’s medium (1950)
  - *Trichoderma* characteristic colony counting
  - Molecular identification of representative isolates
- Molecular analysis
  - DNA isolation
    - DNA isolation at -80°C
  - Multiplex-PCR, tef1, rep-PCR
  - Metagenomic analysis

Oskiera et al. (2017) Biological Control 113: 65-72
**Trichoderma** quantity determination in soil by samples plating and CFU

**Experiment I**

Increased amount of *Trichoderma* in soil after application
Before application less than $10^4$ CFU/gram dry soil

Amount of *Trichoderma* in soil high during lettuce cultivation and also in the next 2 years but decreasing (Experiment I)

1-2 x $10^6$ CFU/gram dry soil

**Experiment II**

1-2 x $10^5$ CFU/gram dry soil

**Experiment III**

1-2 x $10^4$ CFU/gram dry soil - variant T-GRAN + TRS25 + TRS59

1-2 x $10^5$ CFU/gram dry soil - variant T-GRAN + TRS43 + TRS85

Oskiera et al. (2017) Biological Control 113: 65-72
DNA isolation from mineral soil

Comparition of 5 methods of DNA isolation from soil:

1. Fast DNA Spin Kit For Soil (MP Biomedicals, USA) + MP Biomedicals homogenizer
2. Genomic DNA from Soil (Macherey-Nagel, Germany) + MP Biomed. homogenizer
3. SoilMaster DNA Extraction Kit (Epicentre, USA)
4. DNeasy Plant Mini Kit (Qiagen, Germany)
5. CTAB + DNeasy Plant Mini Kit (Aldrich i Cullis, 1993 + Qiagen)

Green frame – best methods
**Trichoderma monitoring in the field soil multiplex-PCR for *T.atroviride* and *T.harzianum***

**Experiment I – first year**

<table>
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<tr>
<th>Fungi ITS 5.8SR/LR6</th>
<th>Trichoderma chi18-5_1a_f/r</th>
<th><em>T. atroviride</em> X18_1F/3R</th>
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</thead>
<tbody>
<tr>
<td><em>Trichoderma</em></td>
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<td></td>
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<tr>
<td><strong>Fungi ITS</strong></td>
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<tr>
<td>5.8SR/LR6</td>
<td>29</td>
<td>33</td>
</tr>
<tr>
<td><em>T. atroviride</em></td>
<td>35</td>
<td>39</td>
</tr>
<tr>
<td><em>T. harzianum</em></td>
<td>48</td>
<td>53</td>
</tr>
<tr>
<td><em>T. harzianum</em></td>
<td>57</td>
<td>61</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Multiplex-PCR</strong></th>
<th><strong>different markers</strong></th>
<th><strong>confirmation of the high amount</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment I</strong></td>
<td><strong>first year</strong></td>
<td><strong>of <em>T. atroviride</em> and <em>T. harzianum</em> in soil after application of biopreparations</strong></td>
</tr>
</tbody>
</table>

Oskiera et al. (2017) Biological Control 113: 65-72
Next Generation Sequencing - Illumina MiSeq

Soil samples - field experiment 1 and 3
- no treatment, carrier, Bioprep1,Bioprep2
- two plots per combination per treatment
- before, 5, 15 weeks after biopreparation application
DNA was isolated from soil samples

PCR amplification:
- fungal **ITS1** region
- bacterial **V4 16S rDNA** region
DNA sequencing **Illumina MiSeq**
**Number of reads** 0.5-0.9 milion per sample
Bioinformatic analysis - counting reads for fungi genera

**MiSeq Illumina** technology is useful to study changes in soil microbial populations as a consequence of biopreparation application - it was possible to estimate the population of fungi at the genera level – species were not distinguished
Conclusions

- Biocontrol *Trichoderma* species persisted at relatively high level in soil during lettuce cultivation and were detected even after two years.
- Multiplex-PCR confirmed the high amount of *T. atroviride* and *T. harzianum* in soil after application of biopreparations.
- Bacterial population changes related *Trichoderma* application were not detected.
- Biopreparation effect on *Fusarium* gives a warning to study carefully dependencies of fungal/microbial communities in the soil towards crops-beneficial soil microorganism management.
Acknowledgments

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