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Organisation de Coopération et de Développement Économiques  
Organisation for Economic Co-operation and Development

17-Dec-2013

English - Or. English

ENVIRONMENT DIRECTORATE  
JOINT MEETING OF THE CHEMICALS COMMITTEE AND  
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

REPORT OF THE 4TH OECD BIOPESTICIDES STEERING GROUP SEMINAR ON  
TRICHODERMA SPP. FOR USE IN PLANT PROTECTION PRODUCTS: SIMILARITIES AND  
DIFFERENCES - ANNEX 4 (PRESENTATIONS)

Series on Pesticides  
No. 74

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This document only contains Annex 4 of the report of the BPSG Seminar. Annex 4 includes slides of all presentations made during the seminar.

The main part of the seminar report, as well as Annexes 1-3, is published under the reference ENV/JM/MONO(2013)25.

## ANNEX 4

### PRESENTATIONS (SLIDES)

**[PPT 1] Presentation on the OECD and the work of OECD-BPSG and general introduction to the seminar on 'Trichoderma'**

*By Jeroen Meeussen, BPSG Chair, European Commission*

**[PPT 2] Plant-beneficial effects of *Trichoderma* and of its genes**

*By Enrique Monte and Rosa Hermosa, Spanish Portuguese Center of Agricultural Research (CIALE), University of Salamanca, 37185 Salamanca, Spain*

**[PPT 3] Overview on the different mode of actions of *Trichoderma* spp.**

*By Maria Isabel Trillas Gay, Fisiologia Vegetal Facultat Biologia, U.B. Barcelona; Spain*

**[PPT 4] *Trichoderma* secondary metabolites and other bioactive molecules: the role in plant protection and biostimulation**

*By Matteo Lorito, Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, Università di Napoli Federico II, Napoli; Italy*

**[PPT 5] Fate and behaviour of *Trichoderma* in the soil and effects on soil microflora**

*By Ilaria Pertot, Fondazione Edmund Mach, FEM-IASMA, Plant Protection Dept., San Michele all'Adige; Italy*

**[PPT 6] Fate of a strain of *Trichoderma atroviride* in soil and effects on the soil microbial communities**

*By Claude Alabouvette, AGRENE, Dijon; France*

**[PPT 7] Taxonomy, characterisation and identification of *Trichoderma*: experience in EU-evaluation and peer review**

*By Kersti Gustafsson, Swedish Chemicals Agency - KemI; Sweden*

**[PPT 8] An overview of regulatory data versus literature data in mammalian toxicology, environmental fate and ecotoxicology in view of a discussion about bridging versus strain/species specific data**

*By Christine Vergnet, French Agency for Food, Environmental and Occupational Health & Safety [ANSES], Paris; France*

**[PPT 9] Task Force I: Experiences with the registration of *Trichoderma harzianum*: active substance and products, in various European countries**

*By Willem Ravensberg, Koppert Biological Systems, Berkel & Rodenrijs; The Netherlands*


**[PPT 10] Task Force II: Experiences with the registration of *Trichoderma viride*: active substance and products, in various European countries**

*By Sara Lamperti, Isagro Ricerca S.r.l., Novara; Italy*

**Presentation on the OECD and the work of OECD-BPSG and general introduction  
to the Seminar on 'Trichoderma'**

**[PPT 1]**

*by Jeroen Meeussen (BPSG Chair, European Commission)*




Seminar on  
" *Trichoderma spp.* for the use in  
Plant Protection Products:  
similarities and differences"

Biopesticides Steering Group

11 June 2012, Paris, France

OECD  OCDE

Seminar on "*Trichoderma spp.* for the use in Plant  
Protection Products: similarities and differences"

- 
- A few words about OECD
  - OECD Work on (Bio)Pesticides
  - Today's seminar: purpose, scope and structure

OECD  OCDE

OECD

OECD: The Organisation for Economic Co-operation and Development



OECD 50

OECD 3 OCDE

The slide features a blue header with the text 'OECD'. Below it, the full name 'OECD: The Organisation for Economic Co-operation and Development' is written. A large, faint 'E' watermark is visible in the background. The central graphic is the OECD 50th anniversary logo, which includes a globe, a bar chart, and the number '50'. At the bottom right, there is a small logo with the text 'OECD 3 OCDE'.

OECD

- Started after **World War II**;
- Transformed in **1961** into the Organisation for Economic Co-operation and Development with trans-Atlantic and then global reach;
- Today the OECD has **34 member countries**;
- **More than 70** developing and transition economies are engaged in working relationships with the OECD (Brazil, China and India).

OECD 4 OCDE

The slide features a blue header with the text 'OECD'. Below it, a list of four bullet points describes the organization's history and current status. A large, faint 'E' watermark is visible in the background. At the bottom right, there is a small logo with the text 'OECD 4 OCDE'.



## OECD - Working Group on Pesticides

The OECD work on **agricultural pesticides** aims to help member countries:

- improve the efficiency of **pesticide control**;
- share the work of **pesticide registration and re-registration**;
- minimise non-tariff **trade barriers**;
- **reduce risks** to human health and the environment.



OECD  7 OCDE

## OECD - Working Group on Pesticides

Working Group on Pesticides:

- Registration Steering Group (RSG)
- Risk Reduction Steering Group (RRSG)
- **BioPesticides Steering Group (BPSG)**

OECD  8 OCDE

## OECD-BPSG

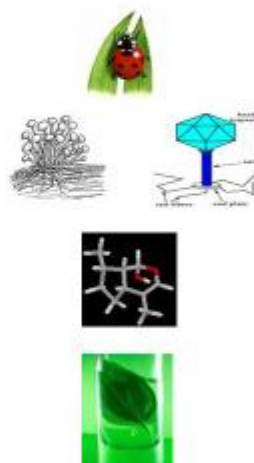
The BioPesticides Steering Group (BPSG) was established by the WGP in 1999 to help member countries:

- to harmonise the methods and approaches used to **assess biological pesticides** and
- to improve the efficiency of **control procedures**.

## OECD-BPSG

### Biological Pesticides:

- Macro-organisms
- Microbial biopesticides
- Semiochemicals
- Plant extracts/Botanicals



## OECD-BPSG

The first tasks of the BPSG consisted of:

- (i) reviewing regulatory **data requirements** for three categories of biopesticides; and
- (ii) developing **formats for dossiers and monographs** for microbials, and pheromones and other semio-chemicals.



OECD  11 OCDE

## OECD-Publications (1)

Registration requirements:

- for **pheromones** (Series on Pesticides, No. 12, 2001);
- for **microbial pesticides** (Series on Pesticides, No. 18, 2003);
- for **invertebrate biocontrol agents/IBCAs** (Series on Pesticides, No. 21, 2004).

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## OECD-Publications (2)

### Dossier

- OECD **Dossier Guidance for Microbials**, 2003  
rev. August 2006.
- OECD **Dossier Guidance for Pheromones and other  
Semi-chemicals**, 2003

### Monograph

- OECD (**Monograph Guidance for Microbials**), 2003  
rev. August 2006.
- OECD **Monograph Guidance for Pheromones and other  
Semi-chemicals**, 2003.

## OECD-BPSG

The BPSG then decided to concentrate its efforts on **science issues** that remain as barriers to harmonisation and work-sharing.



## OECD-Publications (3)

### *"Working Document on the Evaluation of Microbials for Pest Control"*

This document is essentially a set of **examples/case studies** aimed at helping the regulatory authorities to deal with these issues in the assessment of (microbial) biopesticides.

OECD Environment, Health and Safety Publications, Series on Pesticides No. 43, 2008

## OECD-Publications (4)

### Most recent publications:

- Issue Paper on **Microbial Contaminant Limits** for Microbial Pest Control Products (OECD Series on Pesticides No. 65, 2011)
- Guidance to the **Environmental Safety Evaluation** of Microbial Biocontrol Agents (OECD Series on Pesticides No. 67, 2012)

## Workshop on the Regulation of Biopesticides

- *"Workshop on the Regulation of Biopesticides: registration and Communication issues" 15-17 April 2008, EPA, Arlington, USA; OECD Environment, Health and Safety Publications, Series on Pesticides No. 44, 2009*



*The objectives of the workshop were met:*

- To collect input to resolve science issues;
- To improve communication and information exchange;
- To take forward some of the conclusions from REBECA.

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## OECD-Seminars

1. *Report of Seminar on "Identity and Characterisation of micro-organisms"*, OECD Series on Pesticides No. 53, 2010.
2. *Report of Seminar on "The fate in the environment of microbial control agents and their effect on non-target organisms"*, OECD Series on Pesticides No. 64, 2011.
3. *Report of Seminar on "Characterisation and Analyses of Botanicals for the use in Plant Protection Products"*, Publication in preparation

OECD  18 OCDE

## BPSG - Workplan 2009-2012

- Resolve *science issues* associated with registering biopesticides.
- Promote *communication and information exchange* on Biopesticides.



## Seminar on "*Trichoderma spp.* for the use in Plant Protection Products: similarities and differences"

Why was this topic selected?

- A lot of general issues related to micro-organisms are becoming apparent in *Trichoderma spp.*
- Differences between and even within *Trichoderma spp.* can be significant.
- The seminar can contribute to a better understanding of *Trichoderma* and facilitate the registration of biopesticides in general and of this group in particular.



## *Seminar - Objectives*

The objectives of the seminar are:

- **identify key issues** in the area of "*Trichoderma spp.*";
- **exchange information** on national and international activities in the area concerned; and
- **make recommendations** for further actions and/or possible activities for OECD.

## *Seminar - Scope*

Discussion on:

- the **extrapolation** and **bridging of data**;
- **taxonomy** (should strains be compared according to the old classification or should they be considered with the new classification);
- different **mode of actions** and **efficacy**;
- **persistence** and **behaviour** in the environment;
- the fact that **secondary metabolites** may differ for the same strain if applied to different host plants?
- borderline between the use as a **plant protection product**, **fertilizer** and **biostimulation**?

## *Seminar - Structure*

Presentations on:

- **government**, **research** and **stakeholder** experience and perspectives,

followed by discussion after each set of presentations.




## *Seminar - Results*

With the focus on "Trichoderma spp", the goals of this seminar are

1. for participants to **share information** and to **promote a dialogue** on "Trichoderma spp", and
2. to suggest **future work/issue papers** in the field of "Trichoderma spp." related to the approval of 'micro-organisms' in general.

Seminar on "*Trichoderma spp.* for the use in Plant Protection Products: similarities and differences"




I wish you an interesting and  
useful seminar!

Thank you very much for your attention.

**Plant-beneficial effects of *Trichoderma* and of its genes**  
**[PPT 2]**

*Enrique Monte and Rosa Hermosa, Spanish Portuguese Center of Agricultural Research (CIALE),  
University of Salamanca, 37185 Salamanca, Spain*

OECD BPSG Seminar 11 June 2012, Paris





**The 4<sup>th</sup> BioPesticides Steering Group**

Seminar on “*Trichoderma* spp. for the use in Plant Protection Products: similarities and differences”


**“Plant-beneficial effects of *Trichoderma* and of its genes”**

Enrique Monte and Rosa Hermosa  
CIALE, University of Salamanca, Spain  
emv@usal.es

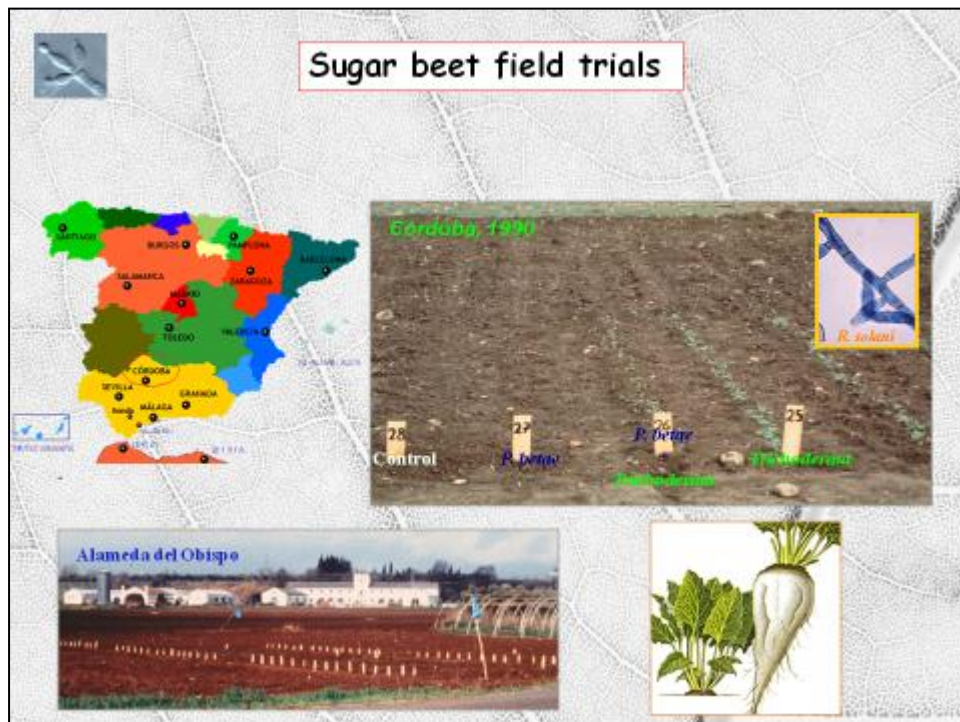


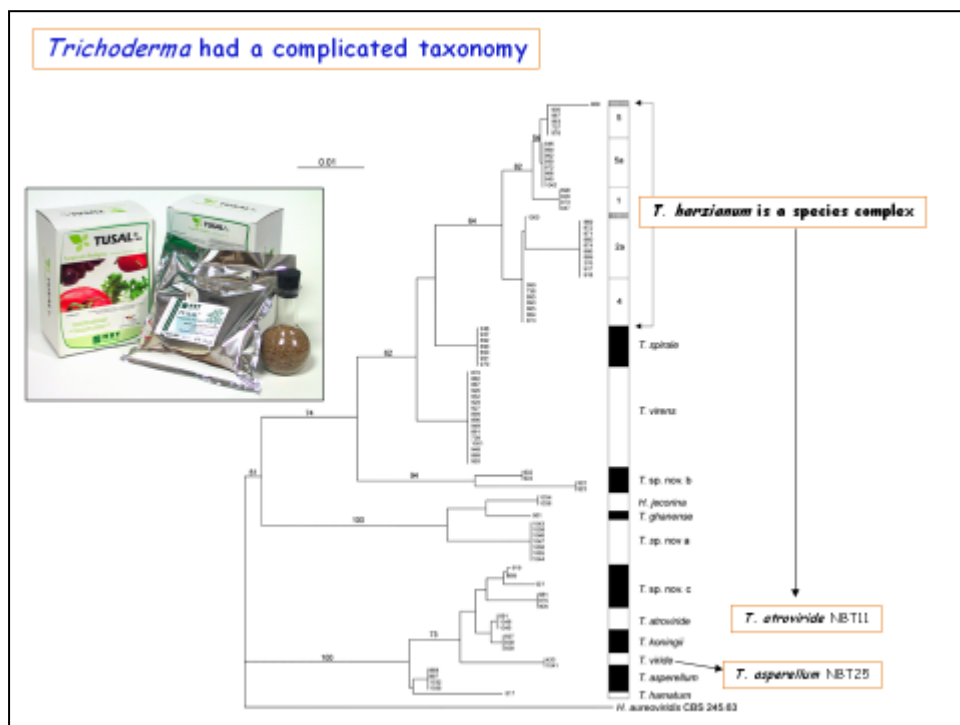
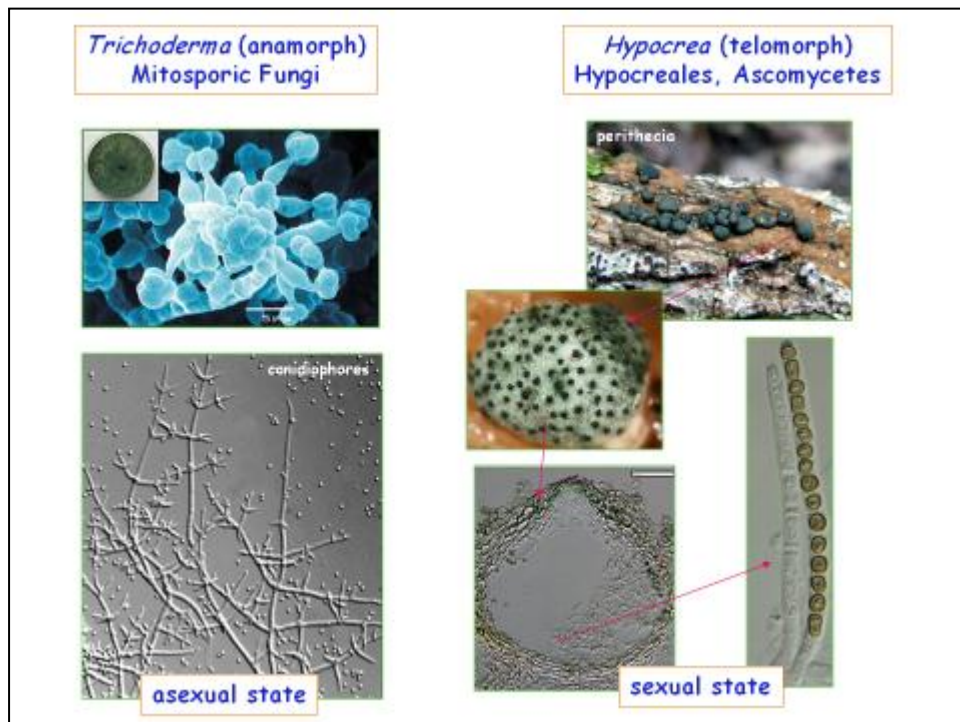
University of Salamanca

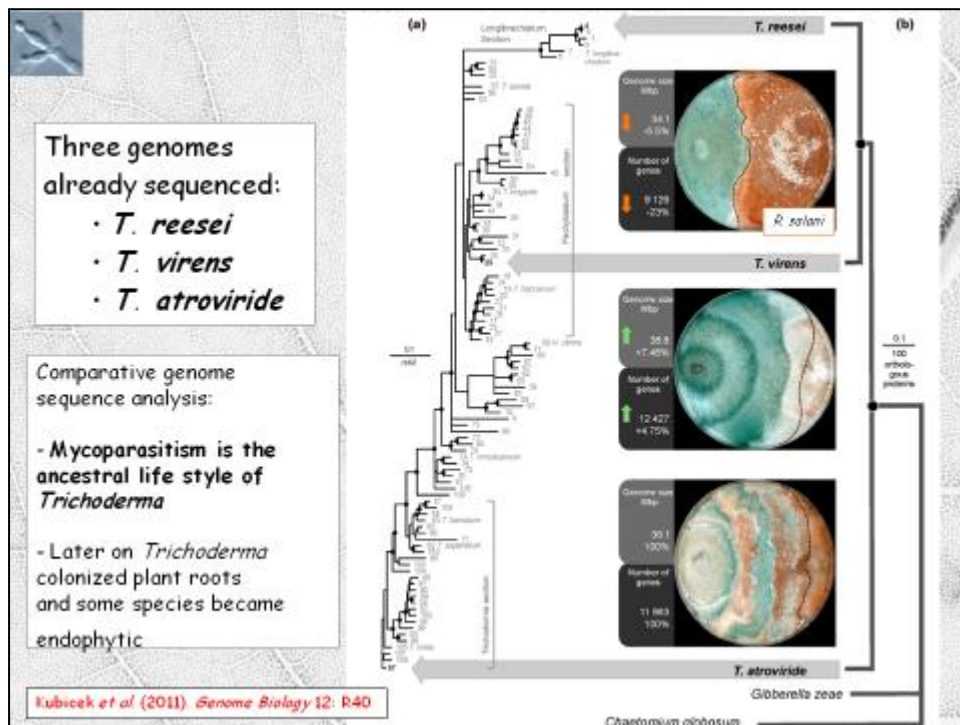
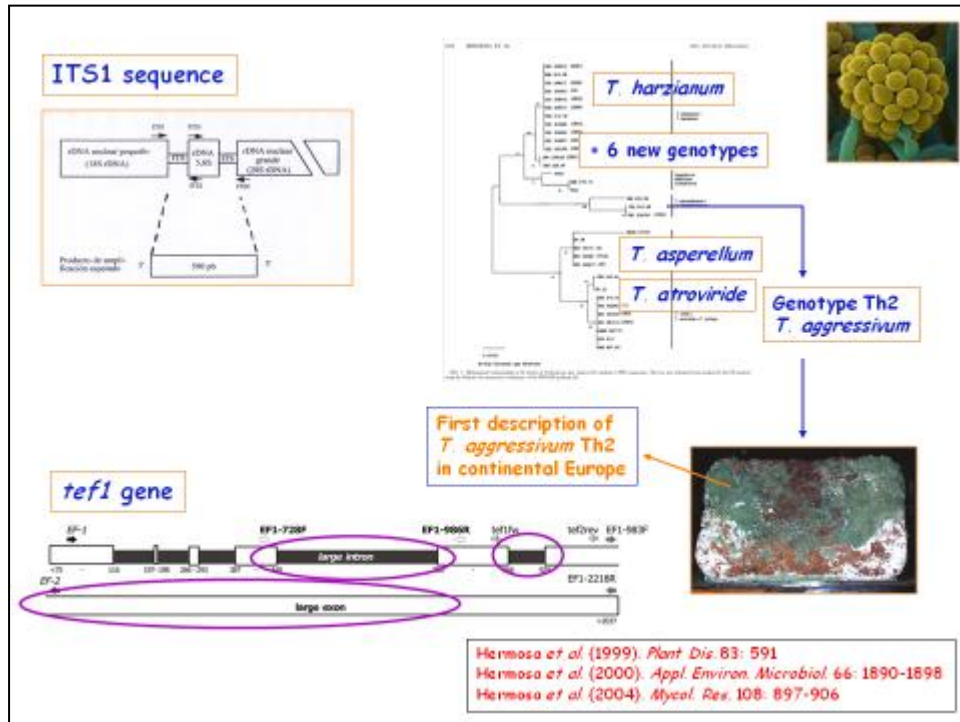
*Trichoderma vs Phoma betae*

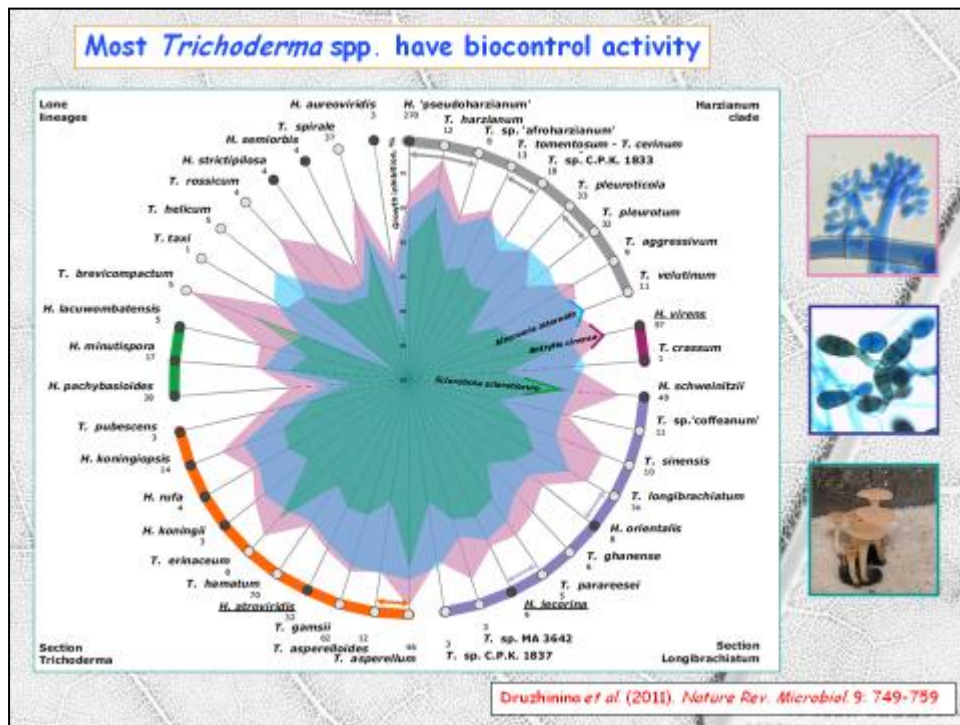


Monte (1986). PhD Thesis. University of Salamanca









**TRICHODERMA displays different mechanisms of action**

**Biocontrol**

**Plant growth promotion, Priming and Defence**

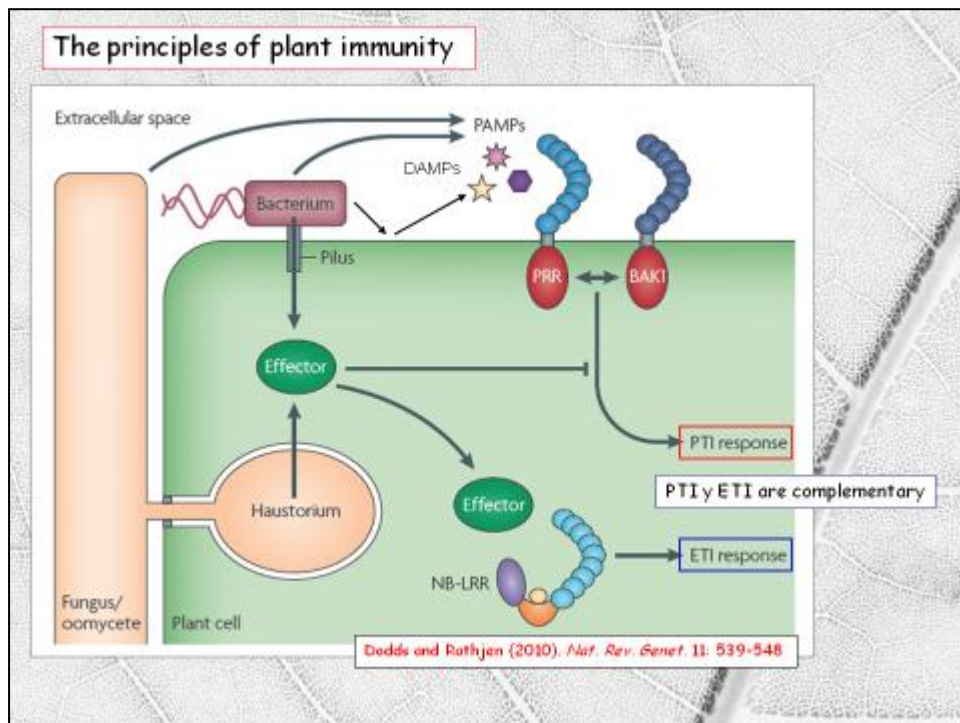
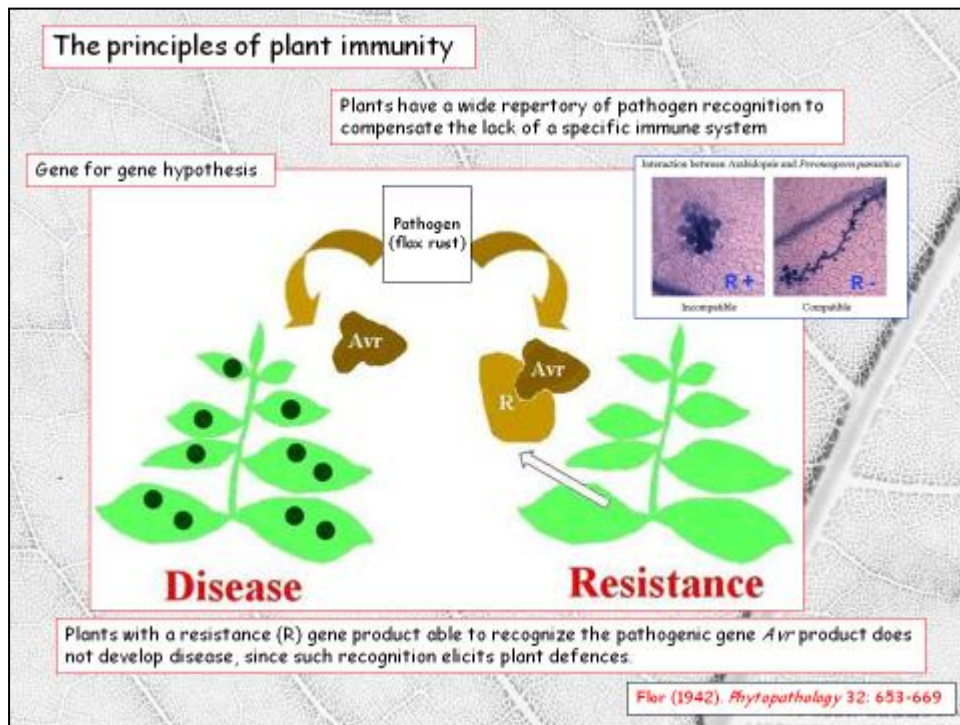
**Courgette**

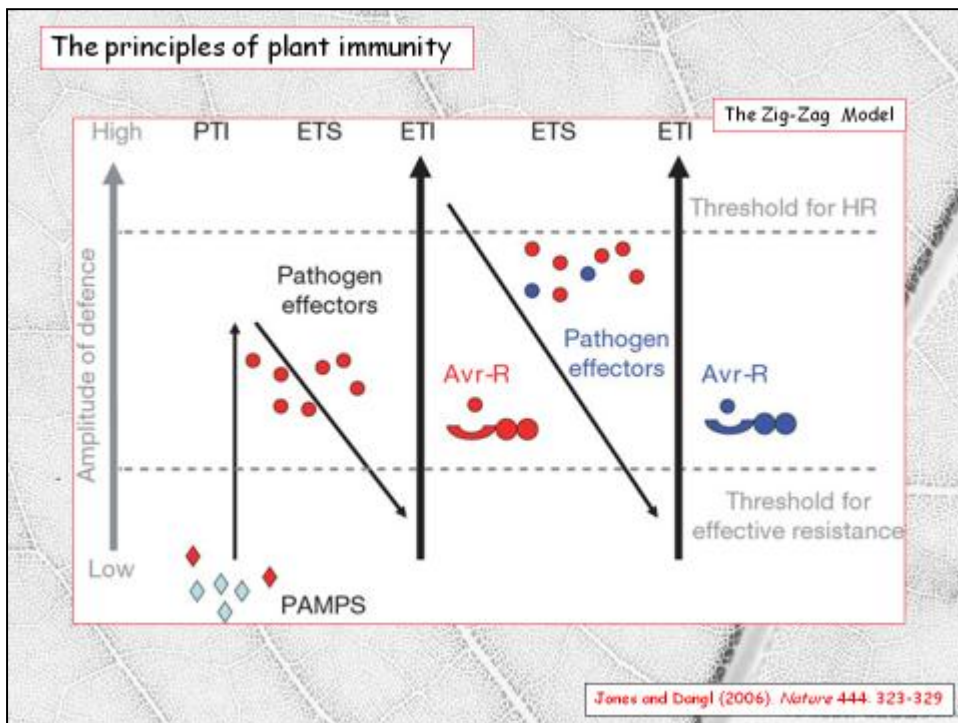
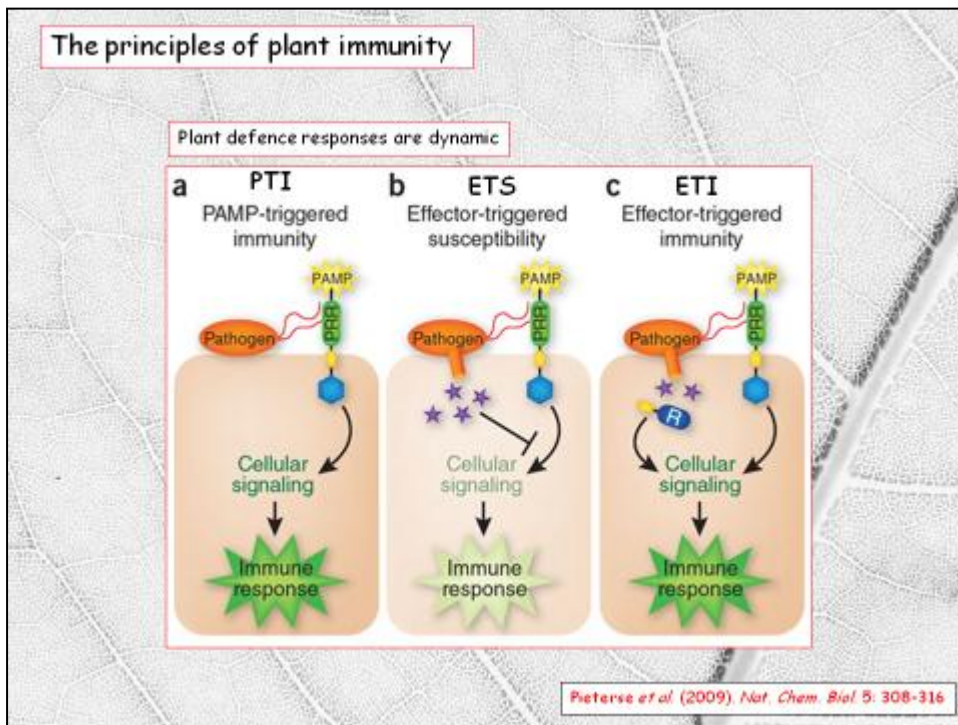
**Tomato crop with *Fusarium* Wilt**

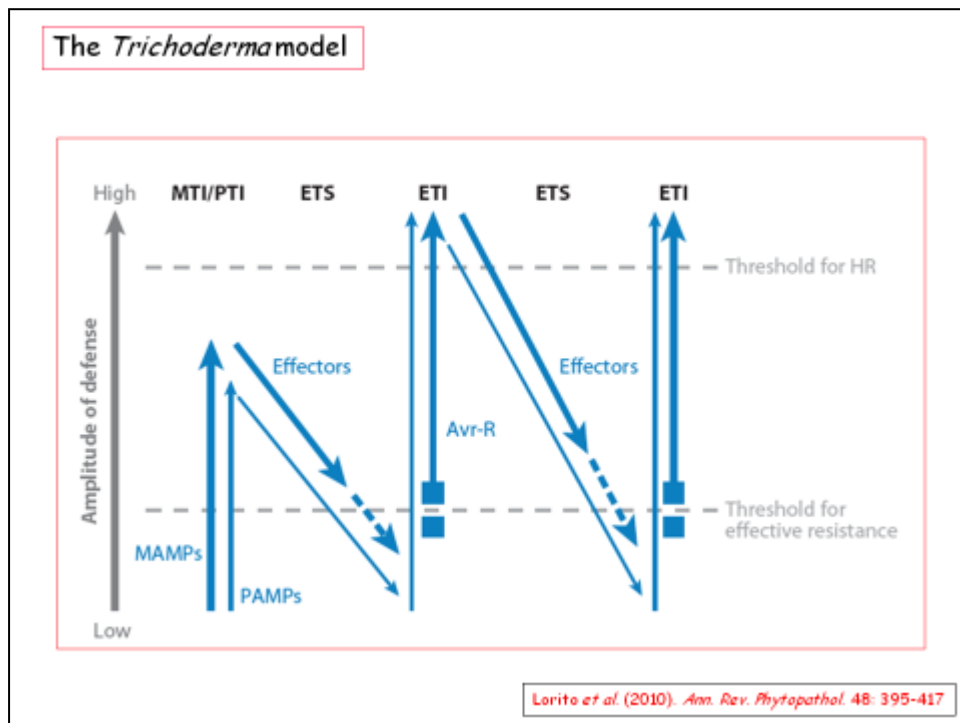
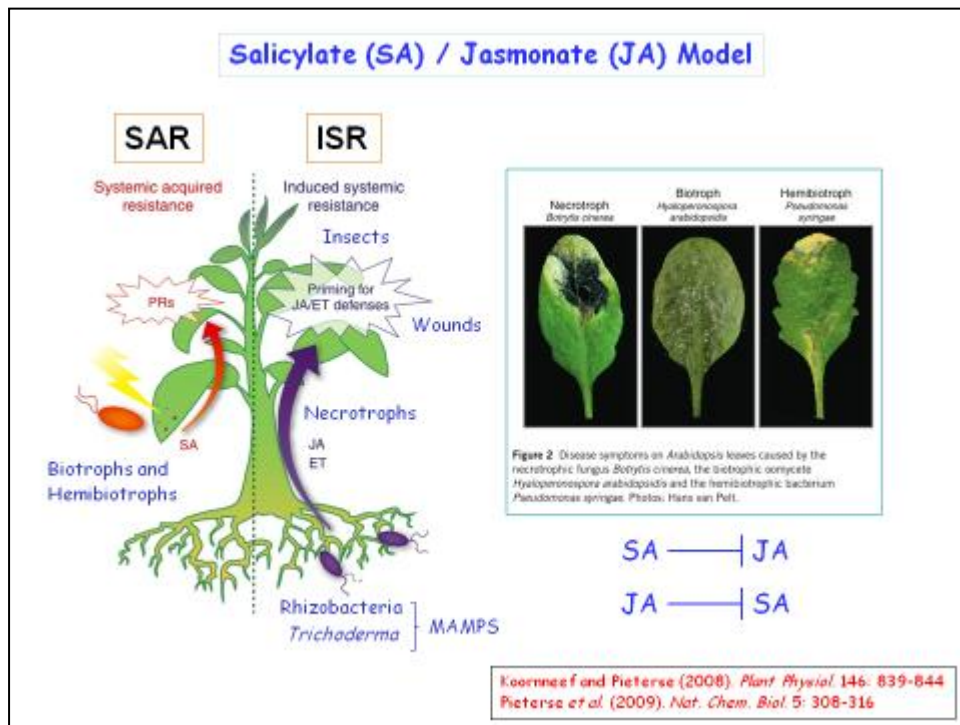
**Trichoderma + 70.000 kg/ha**      **Trichoderma - 56.600 kg/ha**

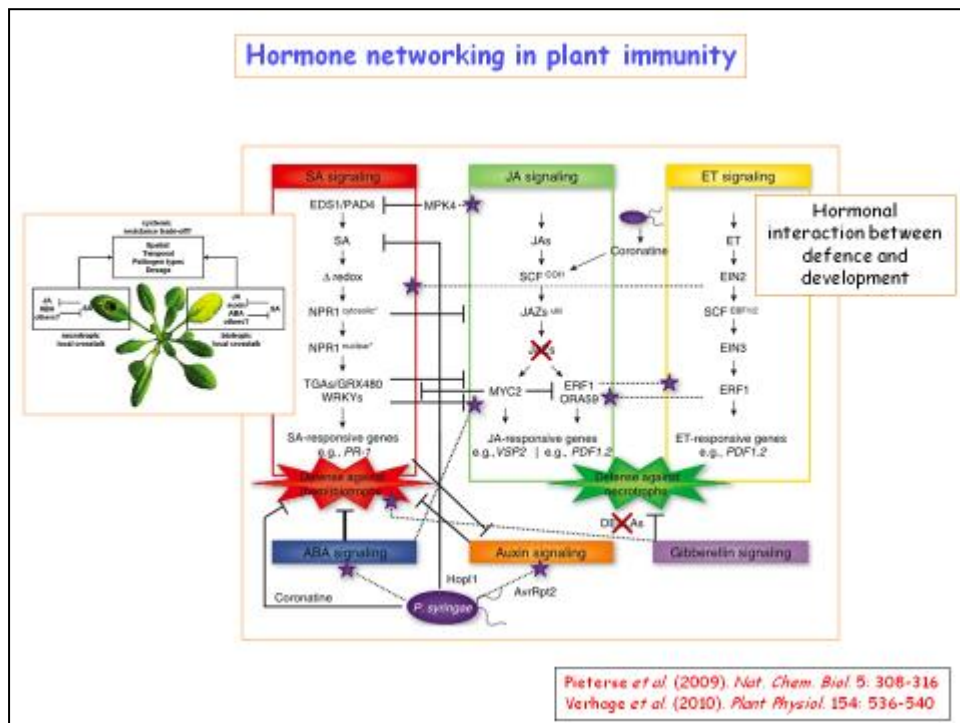
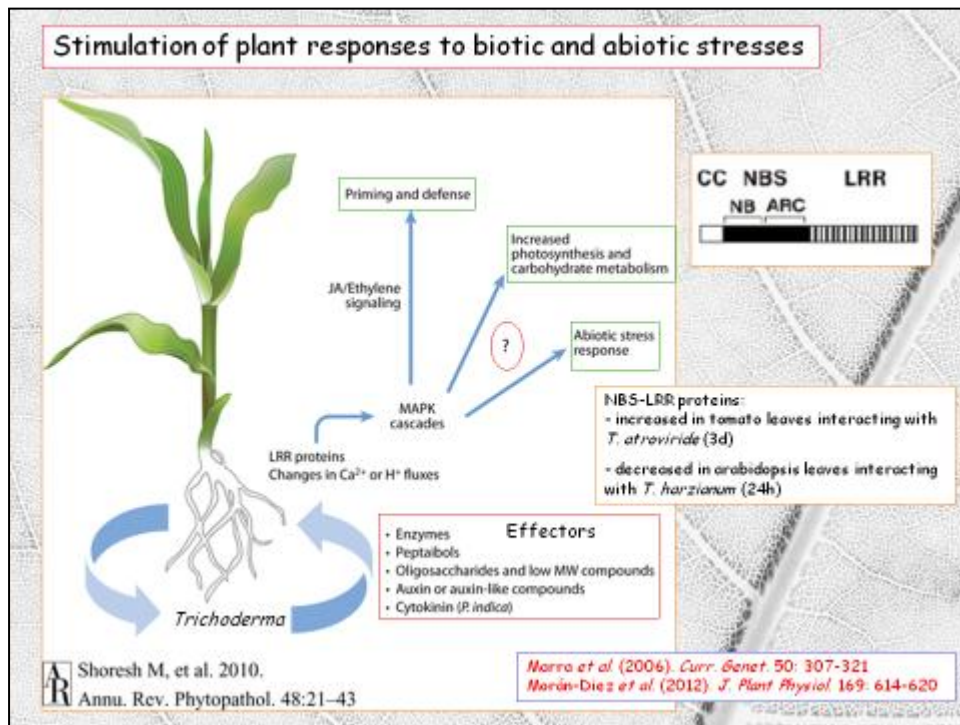
**Mycotrophy** and **Root colonization** images showing Trichoderma interacting with plant roots.

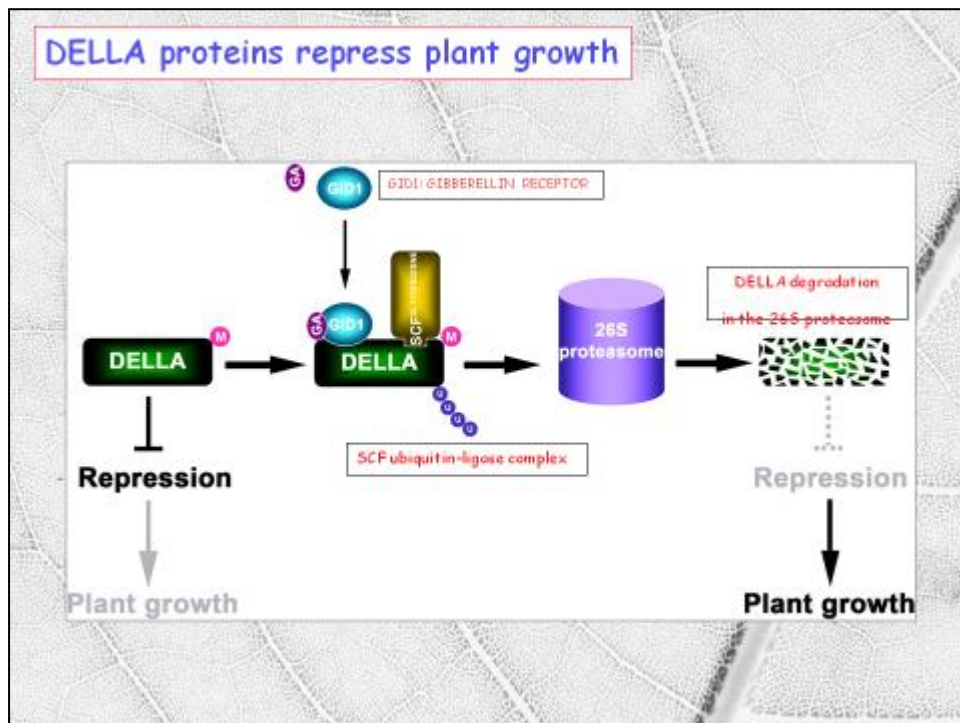
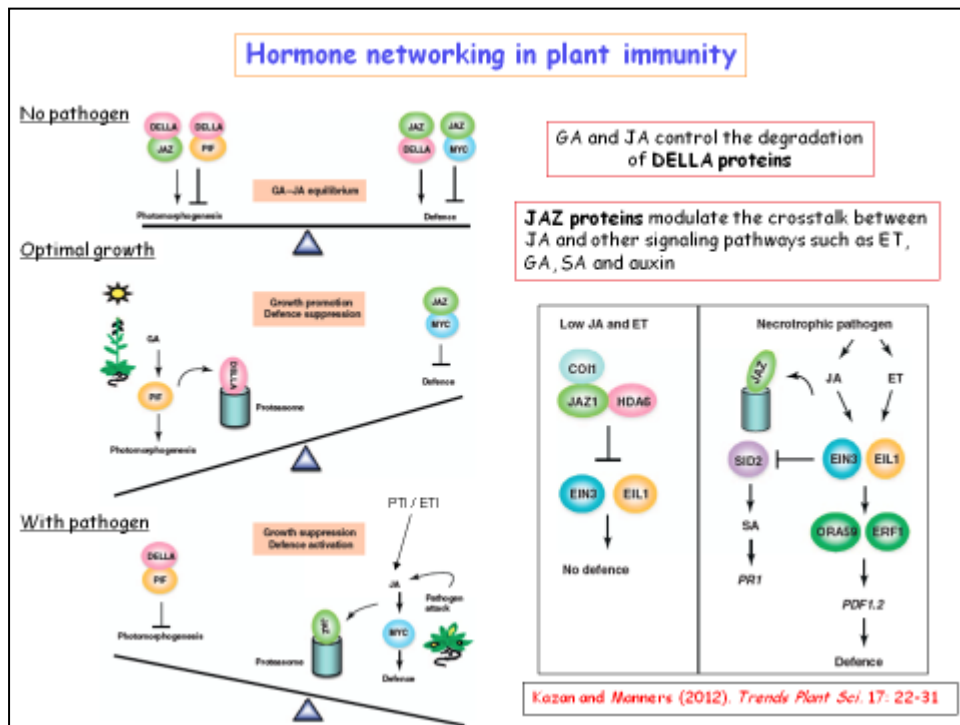
*Harman et al. (2004). Nature Rev. 2: 43-56*  
*Lorito et al. (2010). Ann. Rev. Phytopathol. 48: 395-417*







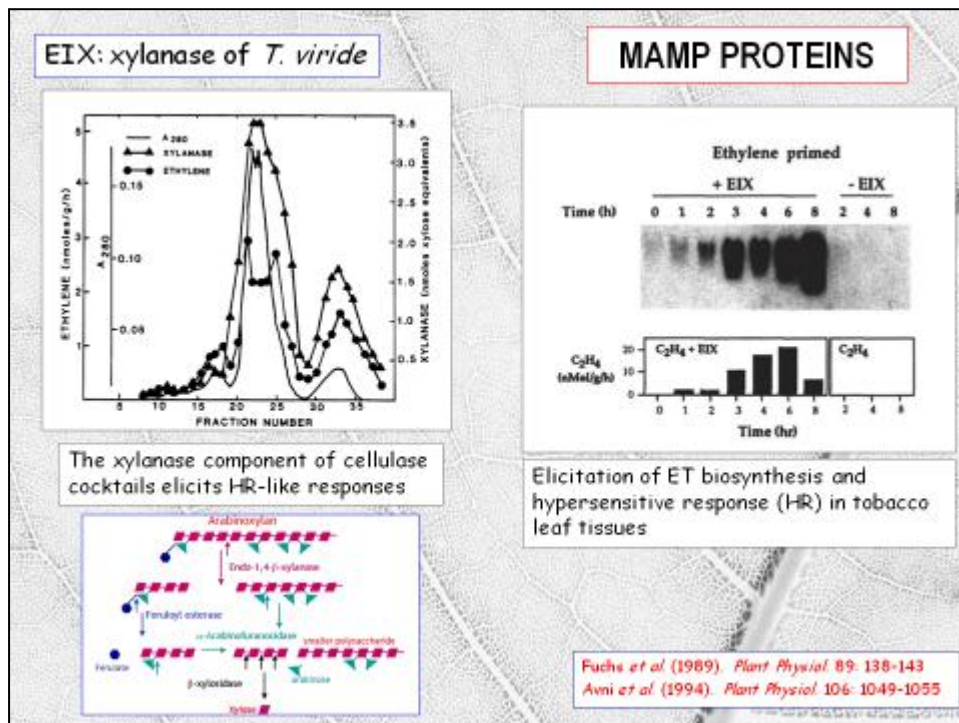




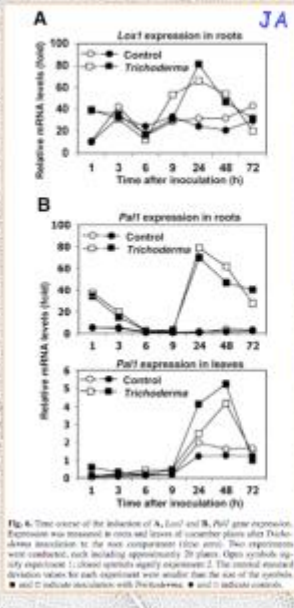
**Table 1.** *Trichoderma* MAMPs currently identified in different species

MAMP/effector	<i>Trichoderma</i> species	Activity	Reference
<b>Proteins</b>			
Xylanase Xyn2/Eix	<i>T. viride</i>	A xylanase that elicits ET biosynthesis and hypersensitive response in tobacco leaf tissues	Rotblat <i>et al.</i> (2002)
Cellulases	<i>T. longibrachiatum</i>	Activated and heat-denatured cellulases elicit melon defences through the activation of the SA and ET signalling pathways, respectively	Martinez <i>et al.</i> (2001)
Cerato-platanins Sm1/Ep11	<i>T. virens/T. atroviride</i>	Hydrophobin-like SSCP orthologues that can induce expression of defence responses in cotton and maize	Djionović <i>et al.</i> (2006), Seidl <i>et al.</i> (2006)
Swollenin TasTwo	<i>T. asperellodes</i>	Expansin-like protein with a cellulose-binding domain capable of stimulating local defence responses in cucumber roots and leaves and affording local protection against <i>B. cinerea</i> and <i>P. syringae</i>	Brotman <i>et al.</i> (2008)
Endopolygalacturonase ThPG1	<i>T. harzianum</i>	Involved in active colonization of tomato root and ISR-like defence in <i>Arabidopsis</i>	Morán-Díez <i>et al.</i> (2009)
<b>Secondary metabolites</b>			
Alamethicin (20mer peptaibol)	<i>T. viride</i>	Elicitation of JA and SA biosynthesis in lima bean	Engelberth <i>et al.</i> (2001)
Trichokonin (20mer peptaibol)	<i>T. pseudokoningii</i>	Induces the production of ROS, the accumulation of phenolic compounds at the application site and virus resistance in tobacco plants through multiple defence signalling pathways	Luo <i>et al.</i> (2010)
18mer peptaibols	<i>T. virens</i>	Elicitation of cucumber systemic defences against <i>P. syringae</i>	Viterbo <i>et al.</i> (2007)
6-Pentyl- $\alpha$ -pyrone, harzianolide and harzianopyridone	Various	Low-concentration metabolite activating plant defence mechanisms and regulating plant growth in pea, tomato and canola	Vinale <i>et al.</i> (2008)

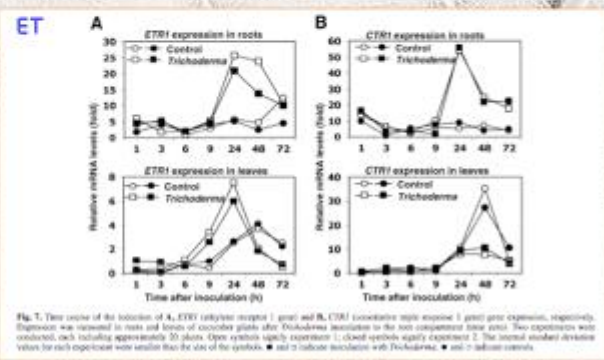
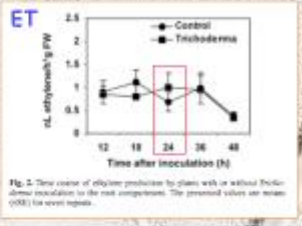
*Hermosa et al. (2012). Microbiology 158: 17-25*



Stimulation of plant defences: increased sensitivity to JA/ET signals



JA/ET signaling pathway are involved in the systemic resistance induced in cucumber by *T. asperellum*



Shoreesh, Yedidia and Chet (2005). *Phytopathology* 95: 76-84

Transcriptomic analysis of gene expression elicited by *T. harzianum* in aerial parts of *Arabidopsis* during the first hours of root colonization

25 d plants

*T. harzianum*

24 h interaction (liquid medium)

+ *Trichoderma*      RNA      RNA      untreated

Arabidopsis microarray

-66 genes (fc 2):

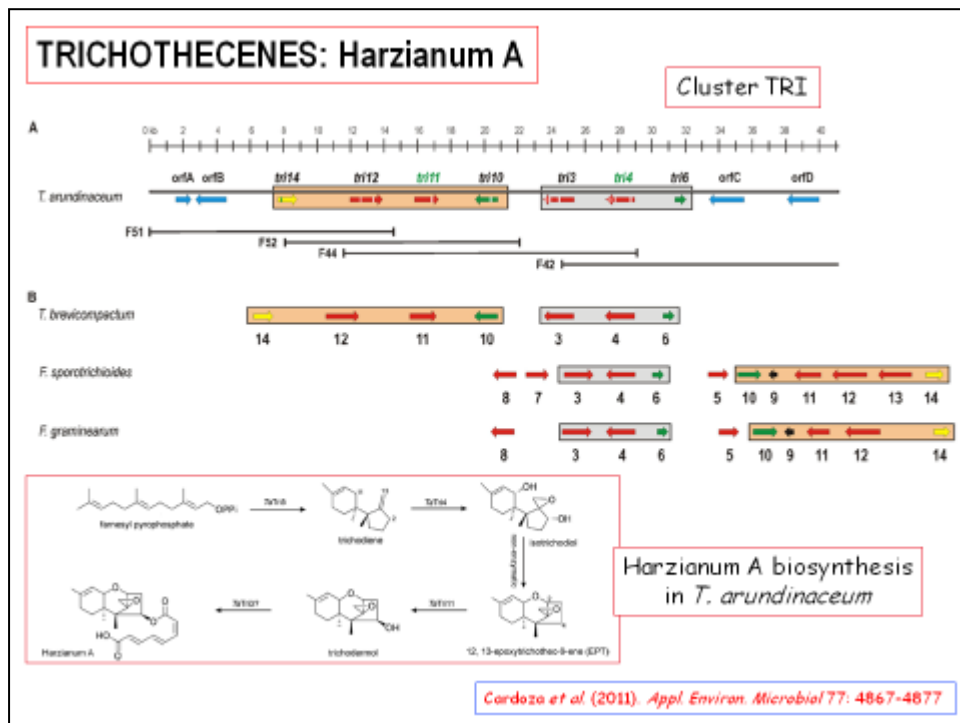
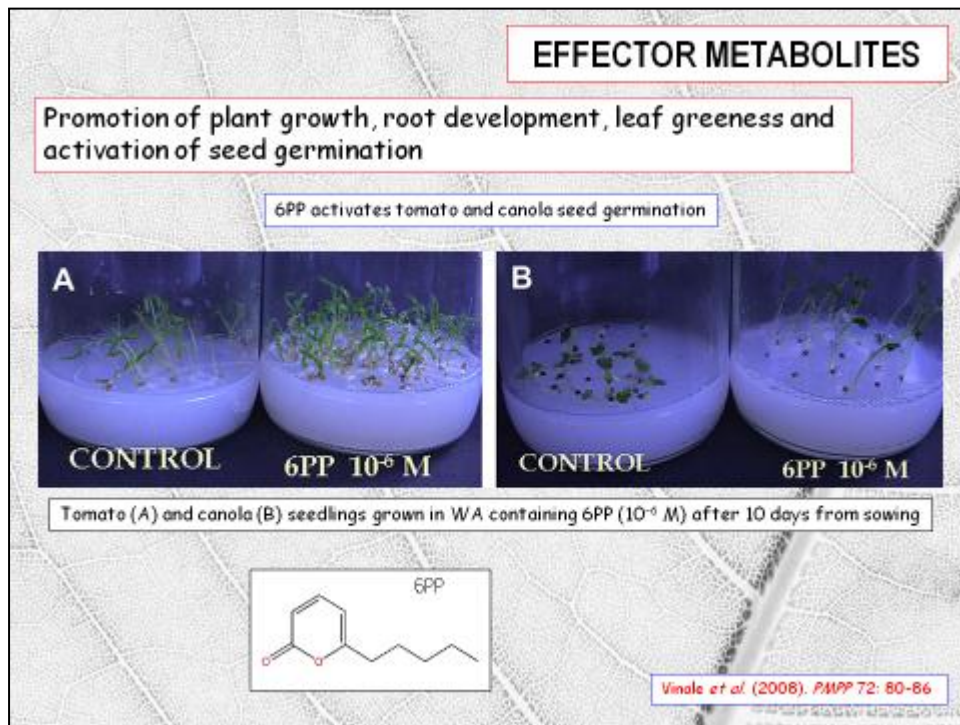
- 36% genes related to metabolism
- 30% genes related to plant responses to stress
- 34% unclassified or unknown proteins

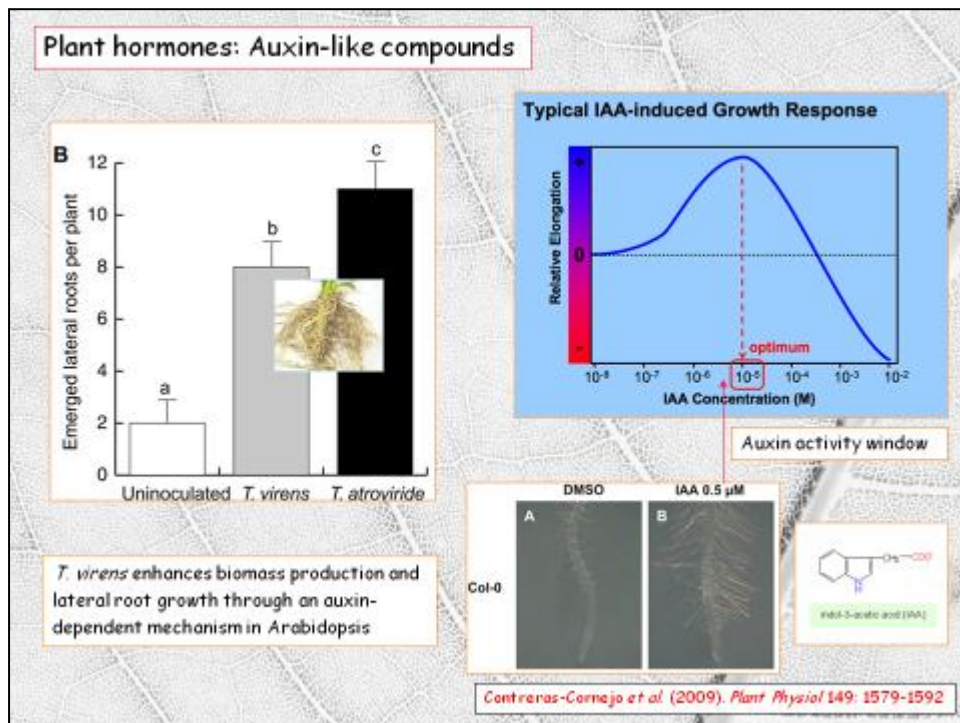
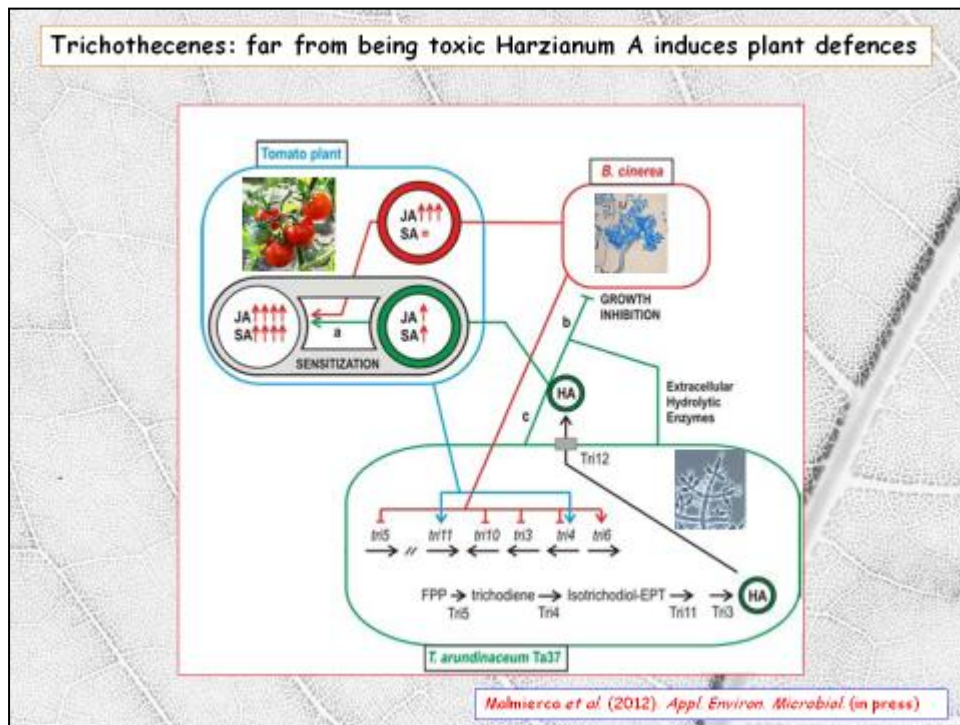
During the first 24 h of *T. harzianum* root colonization, the leaf transcriptome of *Arabidopsis* shows:

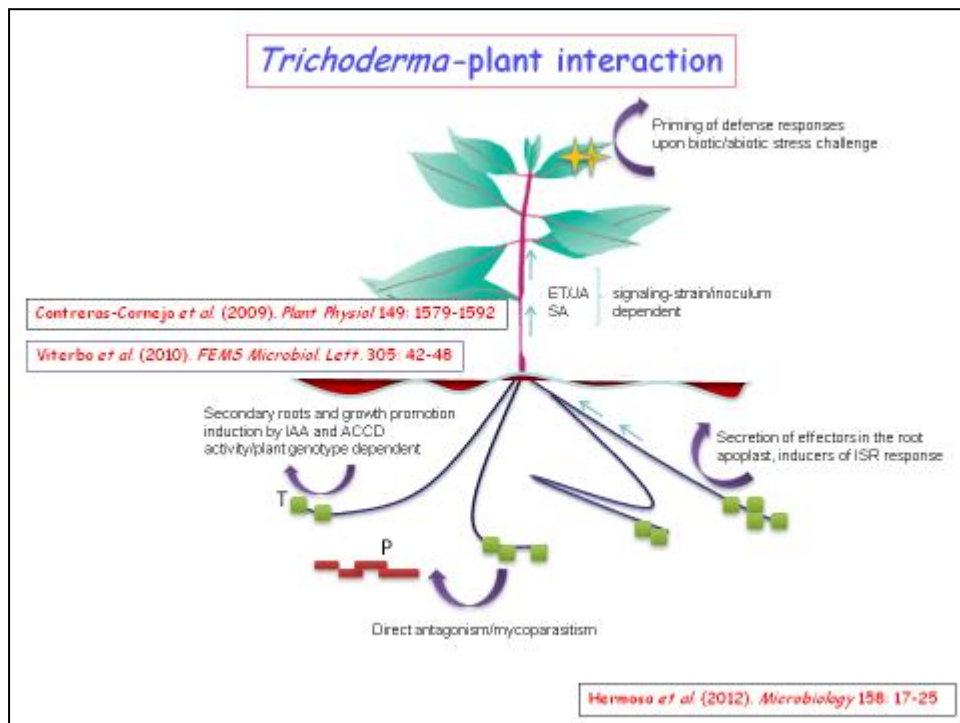
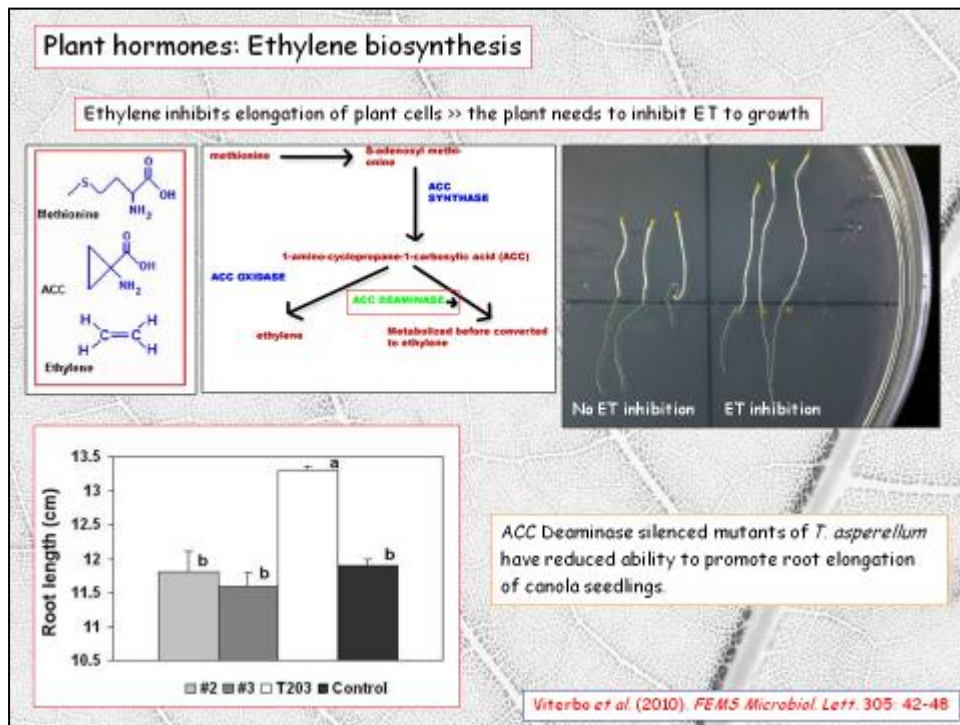
- no modification of ET related genes
- reduction of SA-, JA- and auxin-related genes
- increase of ABA-related genes

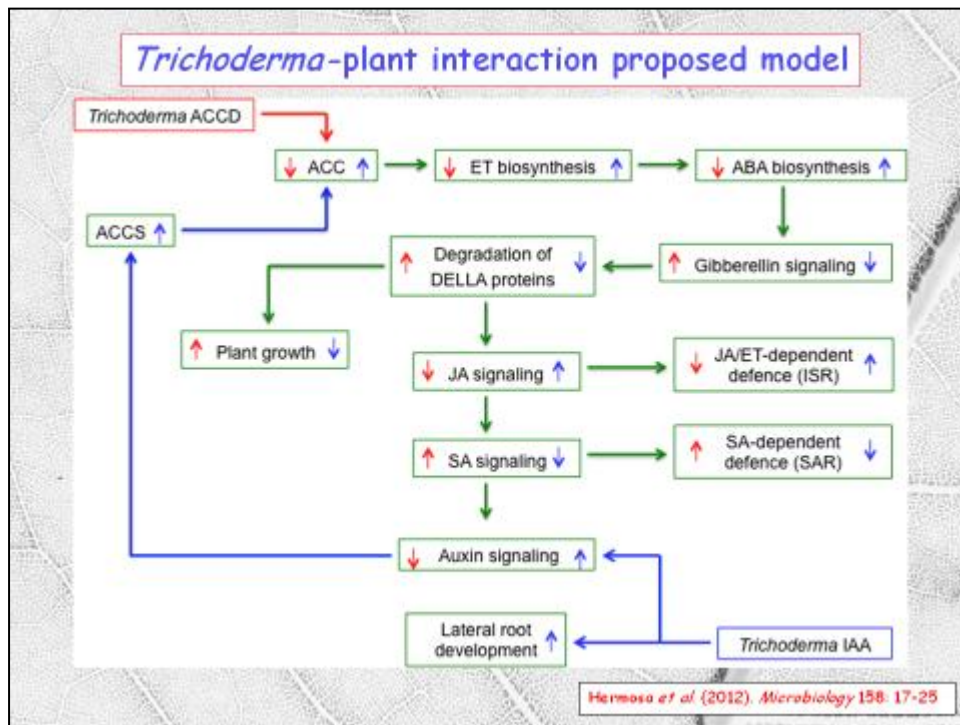
- *Trichoderma* induces abiotic stress resistance
- *Trichoderma* could use in its own benefit the transitory low defenses of plants
- Considering plant immunity networking, SAR/ISR are not absolute models
- Induction of plant responses by *Trichoderma* is a time- and concentration-dependent phenomenon

Morán-Díaz et al (2012). *J. Plant Physiol.* 169: 614-620









### Solubilization of plant nutrients

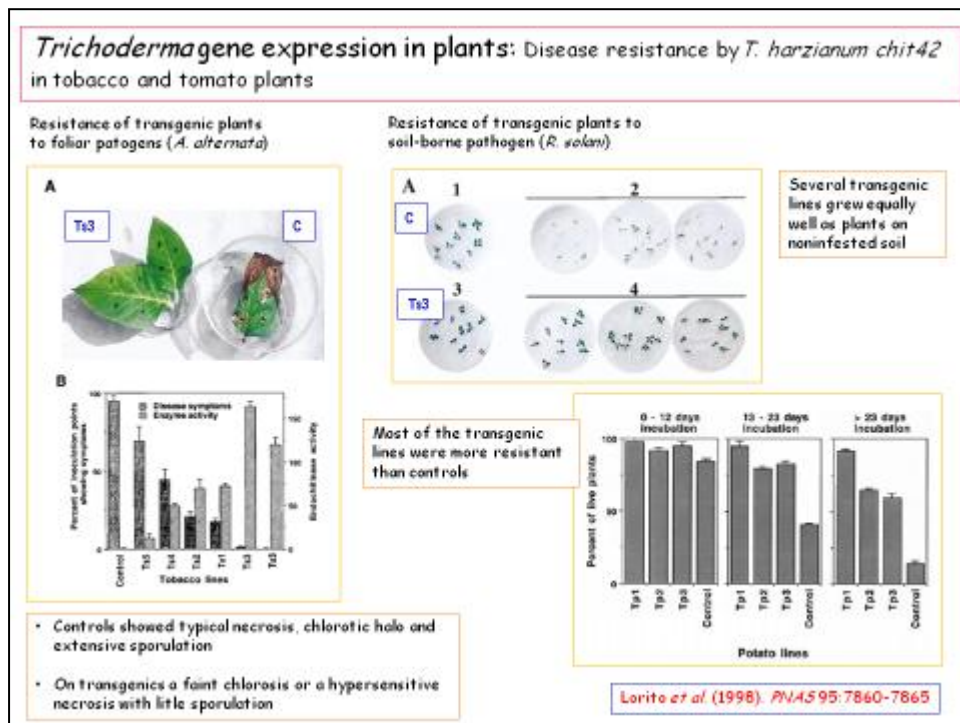
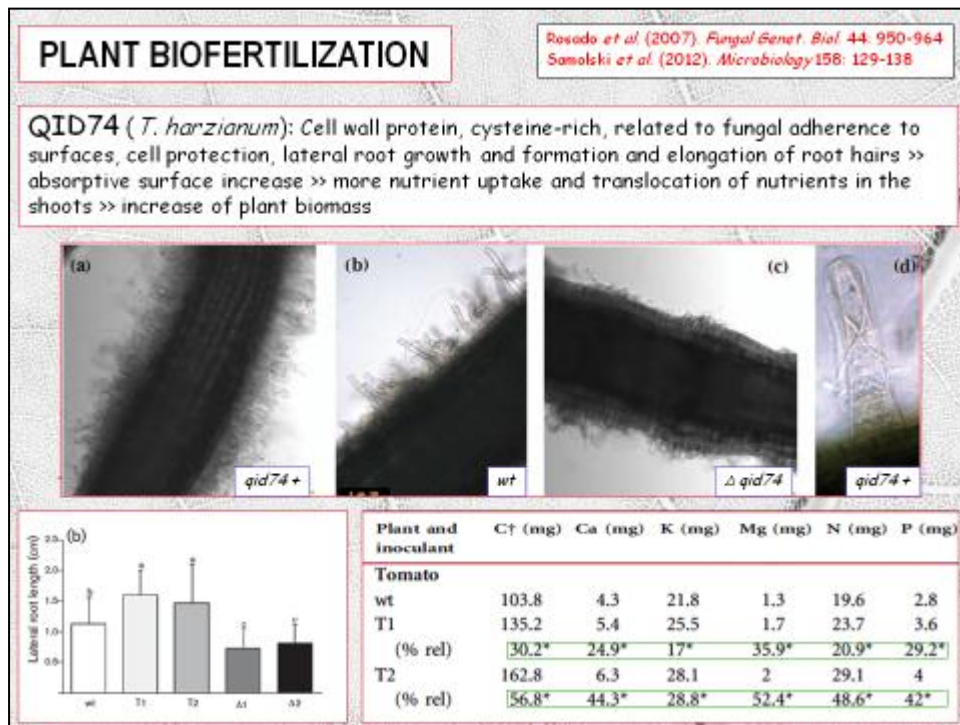
An increase of 90 and 30% in P and Fe concentration respectively, was observed in *T. asperellum* 5 dpi-inoculated plants.

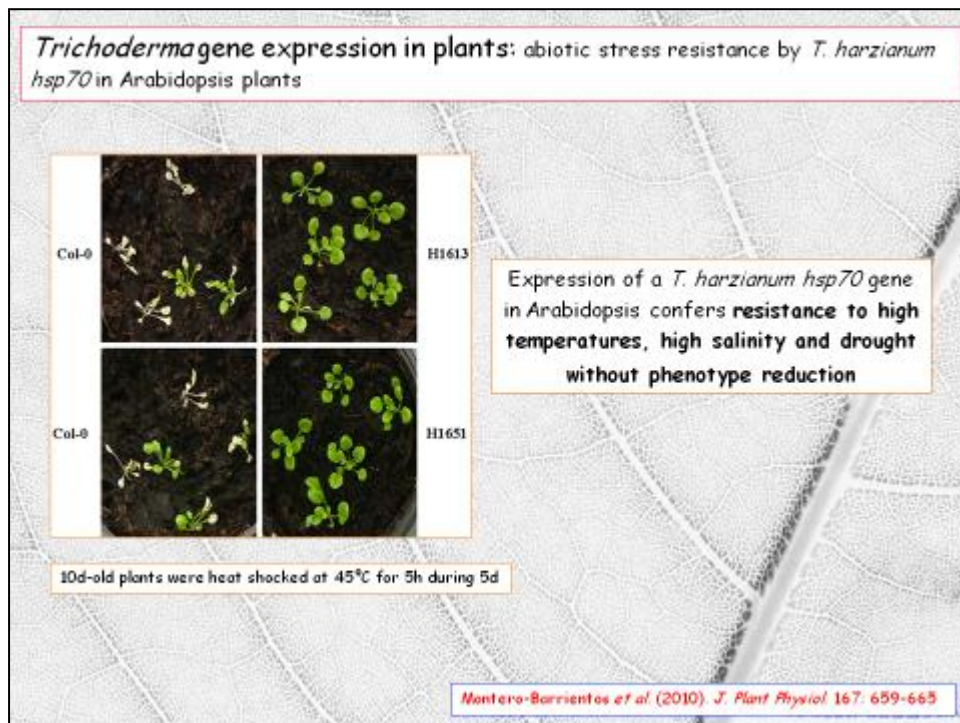
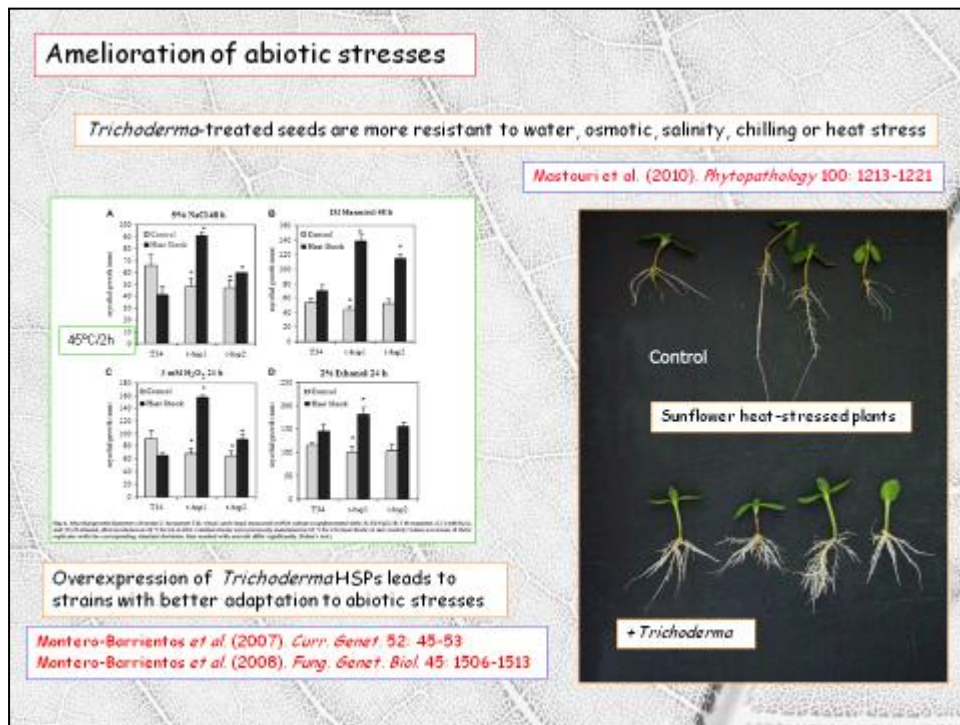
In the shoots of these plants, the 5 dpi-concentration of Zn, P and Mn increased by 25, 30 and 70%, respectively

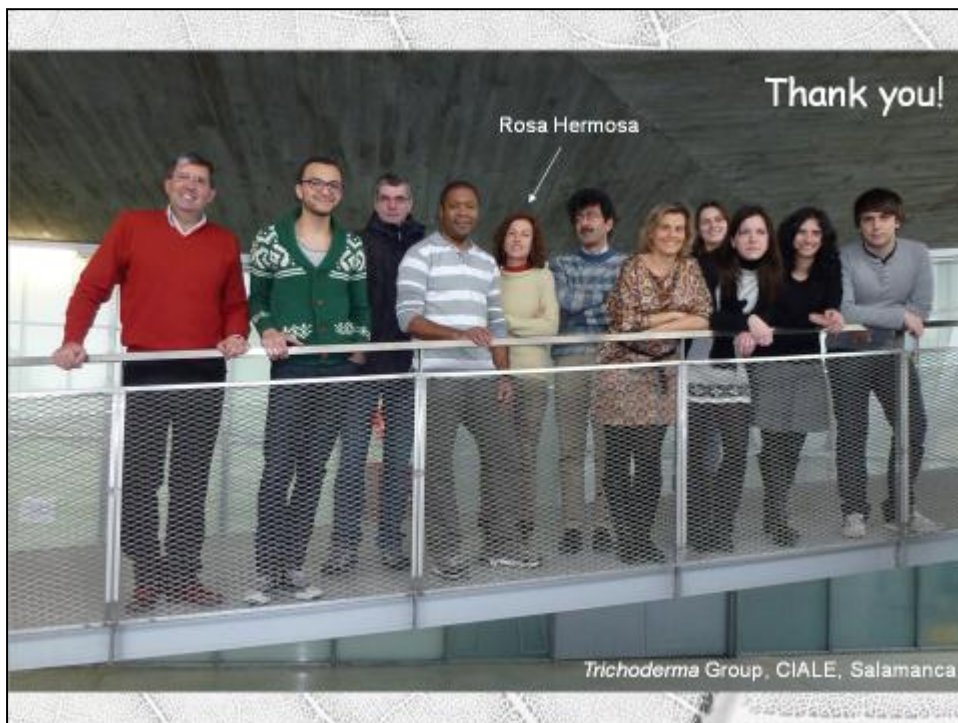
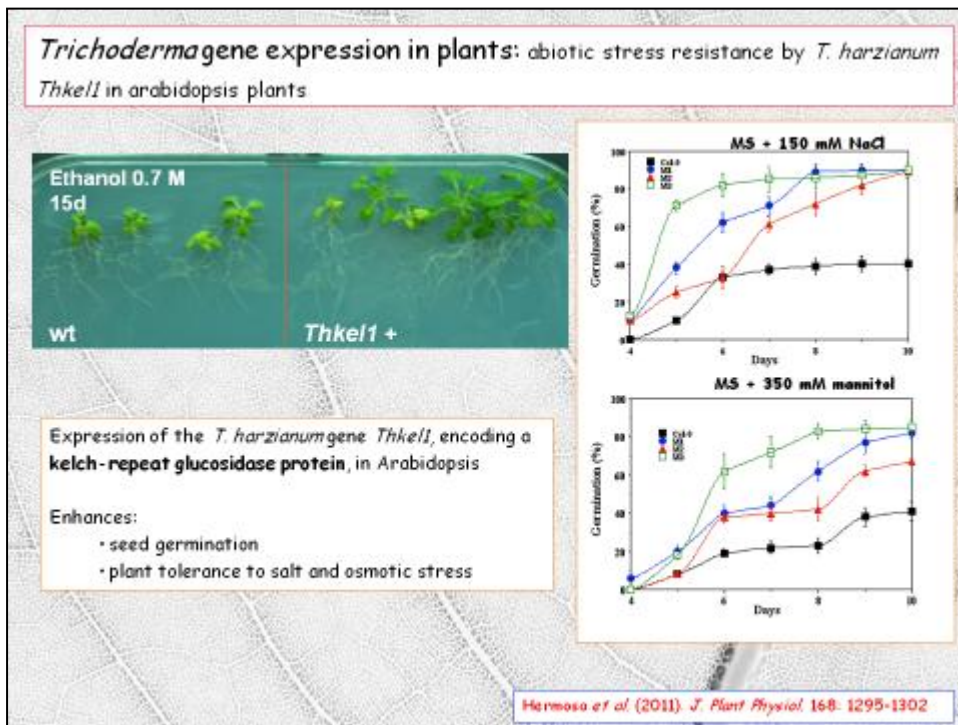
Figure 2. A well-developed root system can more effectively obtain nutrients from the soil.

*Trichoderma-treated hydroponic strawberry*

*Vedidia et al. (2001). Plant Soil 235: 235-241*









**Overview on the different mode of actions of *Trichoderma* spp.**

**[PPT 3]**

*Maria Isabel Trillas Gay (Fisiologia Vegetal Facultat Biologia, U.B. Barcelona; Spain)*



**Overview on the different mode  
of actions of *Trichoderma* spp.**

Dr. M<sup>a</sup> Isabel Trillas, Dr. Guillem Segarra  
Fisiologia Vegetal. Facultat Biologia. Universitat de Barcelona  
Paris, 11 June 2012

  
UNIVERSITAT DE BARCELONA  


OECD BioPesticides Steering Group  
*Trichoderma* spp. for the use in Plant Protection Products: similarities and differences

- 1. INTRODUCTION**
- 2. COLONISATION, ANTIBIOSIS, PARASITISM**
  - 2.1. *Trichoderma* spp. in the plant
  - 2.2. *Trichoderma* spp. competition with pathogens
- 3. NUTRITION**
  - 3.1. Plant growth enhancement
  - 3.2. Competition for nutrients against pathogens
- 4. INDUCTION OF PLANT RESISTANCE**
- 5. CONCLUSIONS**
- 6. REFERENCES**

  
UNIVERSITAT DE BARCELONA  


OECD BioPesticides Steering Group  
*Overview on the different mode of actions of Trichoderma spp.*

## 1. INTRODUCTION

### Toxins and antibiotics

- Microorganisms (*Trichoderma* spp.) are natural producers of **hundreds molecules** which confers competitiveness in front of others microbes.
- The initial trend in Biological Control of *Trichoderma* spp. was focussed in **selecting strains** with high capacity of synthesizing compounds (antibiotics, enzymes, etc.).
  - Selected strains, performed poorly as biocontrol agents in field conditions.



OECD BioPesticides Steering Group  
*Overview on the different mode of actions of Trichoderma spp.*

## 1. INTRODUCTION

### Nutrition

- Microorganisms **compete** against each other for **nutrients** (carbon, nitrogen, phosphorous, iron, and other minor minerals).
- The same molecules (chelates and others) used to make nutrients available are used to make **key nutrients unavailable** for pathogens (growth and virulence).
- After the concept of **PGPRs** (plant growth promoting rhizobacteria) appears the idea of **PGPFs** (plant growth promoting fungi), for *Trichoderma* strains.



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*Overview on the different mode of actions of Trichoderma spp.*

## 1. INTRODUCTION

### Basal resistance vs. induced resistance

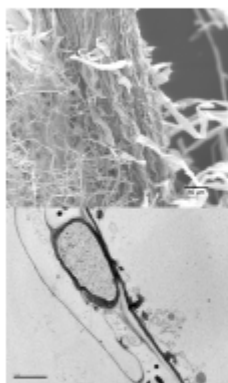
- Plants **spontaneously respond** to pathogens through two main **pathways: SA and JA/ET**.
- **Innate immunity** can be improved by **chemicals** and also **beneficial microorganisms** that **induce a direct plant responses or prime** plants.
- *Trichoderma* spp. first was saw as a **free-living** fungi, later is probed to be also **plant root colonizer**.
- Biological control is not a fight between **good and bad microbes**, it is a **three-way interaction**, playing **plants** an important role.



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Overview on the different mode of actions of *Trichoderma* spp.

## 2. Colonisation, antibiosis, parasitism

### 2.1. *Trichoderma* spp. in the plant



Viterbo and Chet 2006  
Trillas and Segarra 2009  
Samolsky et al. 2012

- Plants release low and high molecular weight compounds, photosynthates, CO<sub>2</sub>, H<sup>+</sup>, etc. selecting specific microbial populations (positive interactions or negative associations).
- *Trichoderma* spp. compete for **root niches** (to exclude pathogens). Most plant pathogens infect root tip, *Trichoderma* spp. too.
  - *T. asperelloides* (T203) colonize root epidermis and penetrates intercellular into the cortical root zone
  - *T. asperellum* (T34) colonisation mostly the root epidermis
- Changes in the **root architecture /extension can be due to:**
  - specific substances:** secreted cysteine-rich protein, QID74 by *T. harzianum* CECT 2413 and hydrophobin TasHyd1 by *T. asperelloides* (T203)
  - plant hormones** CKs, IAA, ET



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Overview on the different mode of actions of *Trichoderma* spp.

## 2. Colonisation, antibiosis, parasitism

### 2.2. *Trichoderma* spp. competition with pathogens

- *Trichoderma* spp. produces more than **100 compounds (SM)**: Anthraquinones, Daucanes, pyrones, Koninginins, Trichodermamides, Viridins, Viridifungins, Nitrogen heterocyclic compounds, Trichodenones and cyclopentenone derivatives, Azaphilones, Harzialactones and derivatives, Butenolides, Trichothecenes, Isocyan metabolites, Setin-like metabolites, Bisorbicillinoids, Diketopiperazines, Ergosterol derivatives, Peptaibols, Cyclonerodiol derivatives, Statins, Heptelidic acid and derivatives, Acoranes and others.
- *Trichoderma* spp. also produces **enzymes**: cellulases (used in recycling cellulosic waste ) and chitinases, glucanases and proteases to parasitize other organisms.
  - Most of these compounds are /species / strain specific:  
Trichotecenes synthesized only by *T. brevicompactum*  
Cellulases mostly by *T. reesei*  
Chitinases mostly expressed by *T. atroviride* and *T. virens*

Nielsen *et al.* 2005,  
Reino *et al.* 2008,  
Mukherjee *et al.* 2012,  
Gruber and Seidl-Seiboth 2012



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Overview on the different mode of actions of *Trichoderma* spp.

## 2. Colonisation, antibiosis, parasitism

### 2.2. *Trichoderma* spp. competition with pathogens

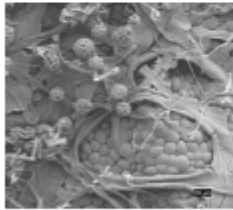
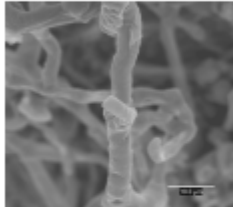
- **Environmental parameters** (i.e. C/N, pH, temperature, presence and type of pathogen) affect synthesis of **SM** and **enzymes**, both production and activity.
- Secondary metabolites of *Trichoderma* spp. can be **useful** or **toxic compounds** for plants and other microorganisms. These compounds can also be absorbed/by organic matter and soil colloids.
- Some of the above mentioned substances could be **toxic** to plants, consumers and the environment.
  - **Toxicological and eco-toxicological tests required for BCAs commercialisation** give more information than analysis of **all** the substances.



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Overview on the different mode of actions of *Trichoderma* spp.

## 2. Colonisation, antibiosis, parasitism

### 2.2. *Trichoderma* spp. competition with pathogens



- *Trichoderma* spp. parasitism is due to the combined action of **recognition** of the cell to parasitize, **attachment / coiling** followed by **lytic activity** to degrade cell walls.
  - **Hyphal coiling and penetration** is a well documented phenomenon of *Trichoderma* spp. (evident in nutrient poor media).
  - **Parasitation of sclerotia** produced by several plant pathogens (*Sclerotium rolfsii*, *Sclerotinia sclerotiorum*, *S. minor*, *Rhizoctonia solani*, *Botrytis cinerea*) has been documented by *Trichoderma* spp.



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Overview on the different mode of actions of *Trichoderma* spp.

## 3. Nutrition

### 3.1 Plant growth enhancement

- Production of **siderophore-like substances (chelation)**, substances segregated by *Trichoderma* spp. as well as other microorganisms (*Pseudomonas* spp.) facilitates the absorption of **iron and other minerals (Ca, Al, Mn, Zn)**.
- Process of **acidification** (release of organic acids or protons) also facilitates the solubilisation of **inorganic Phosphorus**.
- Production of **enzymes** facilitates the mineralization of **organic Phosphorus** (phosphatases, phosphohydrolases).
- Microbial **polymers** (chitin, extracellular proteins) have complexing properties to serve as ligands to trace elements (**Fe, Mn, Zn, Cu, Mo, B, Na, Co, Se**).


Altomare and Tringovska, 2012



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Overview on the different mode of actions of *Trichoderma* spp.


### 3. Nutrition

#### 3.1 Plant growth enhancement



Yedidia et al. 2001,  
Benitez et al. 2004,  
Trillas and Segarra 2009,  
Altomare and Tringovska, 2012

- Isolation and characterisation of siderophores produced by different *Trichoderma* strains have been done: *T. harzianum* 1295-22 (T22), *Trichoderma virens*, strain ATCC 24290.
- Mobilisation and mineral uptake by plants treated with *Trichoderma* spp. is well documented:
  - *T. harzianum* (T22) increase Fe, Mn, Zn, Cu in cucumber plants and not in beans plants (mobilisation and uptake would depend on strain and experimental conditions).
  - *T. asperellum* (T34) increase the contents, in tomato plants, of Fe, S, Cu, Si and B and *T. asperelloides* (T203) increase the content in shoots of Zn, P and Mn and in roots of Cu, P, Fe, Zn, Mn, Na.

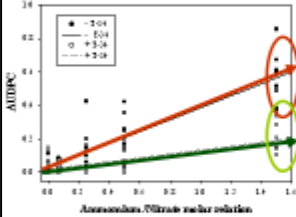


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Overview on the different mode of actions of *Trichoderma* spp.


### 3. Nutrition

#### 3.2. Competition for nutrients against pathogens



Lynch and Hobbie, 1979,  
Harman et al. 1980,  
Segarra et al. 2010,  
Borrero et al. 2012

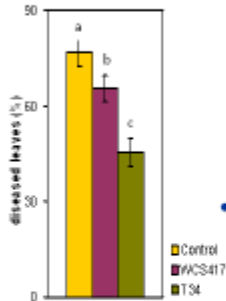
- Iron deprivation limits the effectiveness of *T. hamatum* (isolated from *Rhizoctonia* suppressive soil), and *T. asperellum* (T34), as well as the pathogenicity of *Fusarium* spp.
- *T. asperellum* (T34) spp. compete against *F. oxysporum* f.sp. *lycopersici* for iron and other minerals, as well as nitrogen source, reducing growth and/or virulence of the pathogen.
- Suppression of minor plant pathogens (detrimental but do not cause apparent symptoms of disease) could also enhance plant growth and health.



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#### 4. Induction of plant resistance



Bigirimana *et al.* 1997,  
Yedidia *et al.* 2003,  
Harman *et al.* 2004,  
Segarra *et al.* 2009



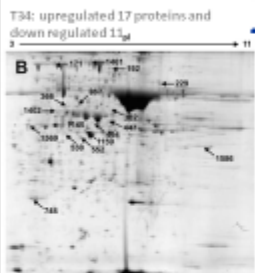
- **Systemic resistance**, *Trichoderma* spp. in the roots diminish disease in the leaves.
  - *T. asperellum* (T34) reduced disease caused by: *P. syringae* pv. *tomato* and pv. *lachrymans*, *Plectosphaerella cucumerina* and *Hyaloperonospora parasitica*
  - *T. asperelloides* (T203) *P. syringae* pv. *lachrymans*
- **Direct activation** of defences is costly and could cause yield reduction.
 

**Priming** entail low-cost metabolic changes. These may be small and difficult to detect experimentally but enables plant to respond more rapidly and strongly after pathogen attacked.

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Overview on the different mode of actions of *Trichoderma* spp.

#### 4. Induction of plant resistance



Harman *et al.* 2004,  
Alfano *et al.* 2007,  
Segarra *et al.* 2007,  
Segarra *et al.* 2009,  
Trillas and Segarra 2009



- Induction of resistance is a well documented phenomena
  - *T. asperelloides* (T203), increase PAL and HPL directly.
  - *T. atroviride* (P1), up regulate NBS-LRR type resistance protein and PR10.
  - *T. harzinaum* (T22), produce an increase of glucanases, chitinases and priming for some of these enzymes in the presence of the pathogen.
  - *T. hamatum* (T382), induce genes related to biotic and abiotic stress, RNA, DNA, and protein metabolism.
  - *T. asperellum* (T34), at high concentration induce synthesis of proteins involved in: ROS scavenging, stress response, isoprenoid and ET biosynthesis, photosynthesis, photorespiration and carbohydrate metabolism.
- *T. asperellum* (T34), at low concentration primes the plant to respond by blocking entrance to biotrophic leaf pathogen by synthesis of callose and to express *LOX 2* after Me-JA application.

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### 4. Induction of plant resistance

**SAR** SA + PR { Biotrophic Pathogens { PGPR Mycorrhiza { PGPF (Trichoderma)

**ISR** JA + ET { Necrotrophic Pathogens { PGPR Mycorrhiza Wounds Herbivores Insects { PGPF (Trichoderma)

DIRECT EFFECTS and/or PRIMING

Other plant hormones involved: **Auxins, CKs, ABA,**  
 Small **RNA**: up + down regulated response to pathogens  
**Volatile compounds**: VOCs (volatile organic compounds, Me JA, MeSA) and GLV (green leaf volatile): plant -plant and plant herbivores communication, plant-pathogen? Plant -PGPR/PGPF?




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Overview on the different mode of actions of *Trichoderma* spp.

### 5. Conclusions

*Trichoderma* spp. is a **natural antagonist** of fungal pathogens and probably offers the greatest prospect as **broad-spectrum biocontrol agent** (i.e. *Fusarium oysporum*, *Rhizoctonia solani*, *Botrytis cinerea*, *Sclerotinia* spp. *Pythium* spp. ...).

*Trichoderma* spp. biocontrol agents use a **variety of mechanisms of action** against pathogens: compete for space, nutrients, produce secondary metabolites and enzymes that may differ according the **strain**, the **pathogen** and the **environment**.

In plants, selected strains of *Trichoderma* spp. **induce systemic resistance** (ISR, SAR, direct effects or priming) and **promote growth**.



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Overview on the different mode of actions of *Trichoderma* spp.

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THANK YOU FOR YOUR ATTENTION

Thanks to the team of Biocontrol Technologies, S.L.



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Overview on the different mode of actions of *Trichoderma* spp.


***Trichoderma* secondary metabolites and other bioactive molecules:  
the role in plant protection and biostimulation**

[PPT 4]

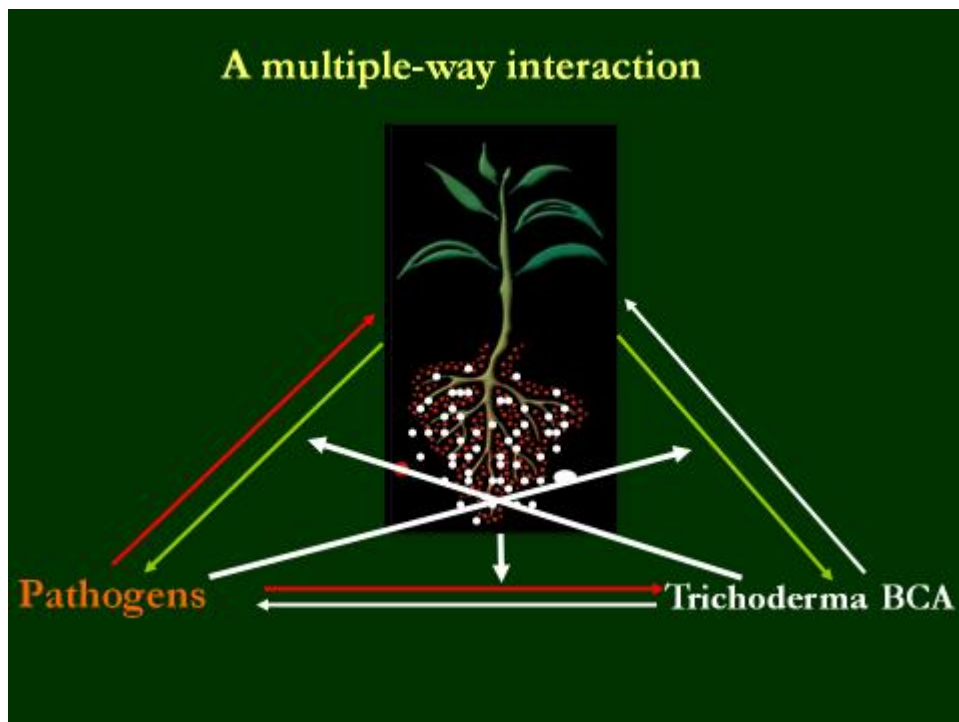
Matteo Lorito (Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, Università di Napoli  
Frederico II, Napoli; Italy)

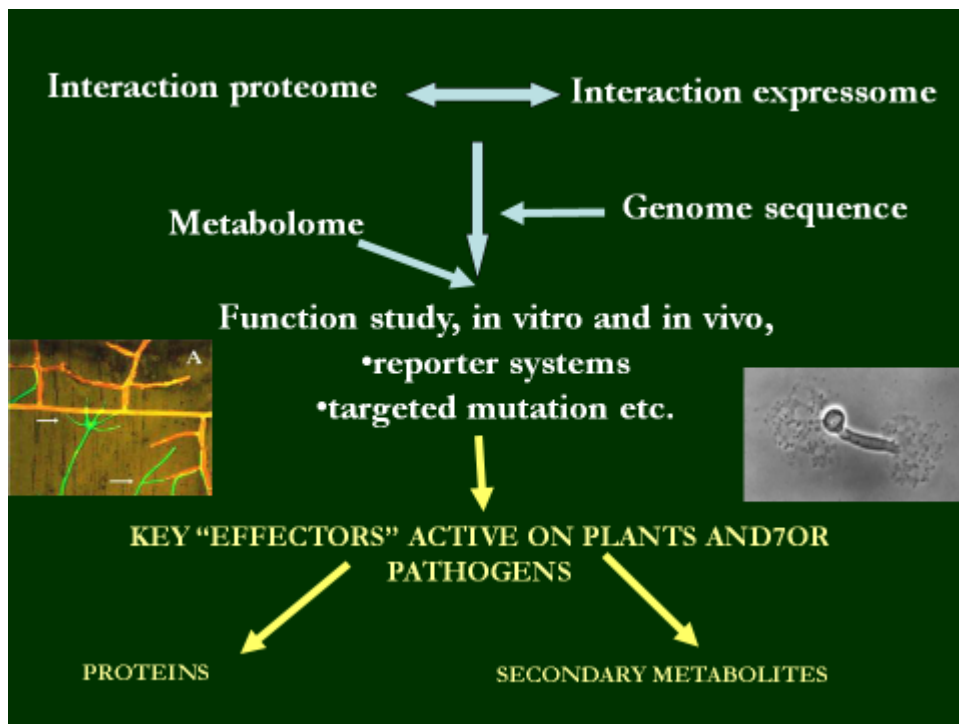
***Trichoderma* secondary metabolites and other bioactive molecules:  
the role in plant protection and biostimulation**

**MATTEO LORITO**  
University of Naples - Faculty of Agriculture  
lorito@unina.it



World wide use of *Trichoderma* in agriculture





### ***Trichoderma* factors affecting the plant**

- a variety of small and still uncharacterized proteins also having domain similarity with pathogen factors
- different hydrophobins such as Sm1/ep11 and Hytra1 (up to 10 class-II hydrophobin genes, while most Ascomycetes have 1 or 2)
- swollenins and expansin-like proteins
- various enzymes and some secondary metabolites

## Secondary metabolites

Heterogeneous group of chemically different natural compounds, in general of a molecular weight < 3000 D

### Role of secondary metabolites

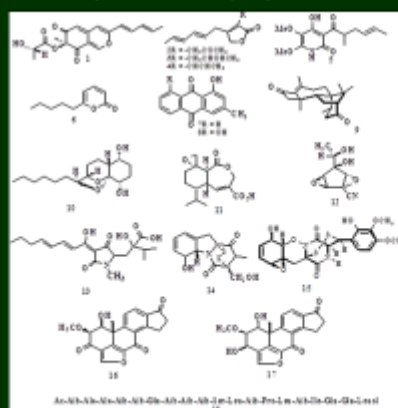
- i) competitive weapons used against bacteria, fungi, plants, insects and animals;
- ii) metal transporting agents;
- iii) agents of symbiosis between microbes and plants, nematodes, insects;
- iv) sexual hormones.

They include antibiotics = natural products capable of inhibiting or killing their microbial competitors

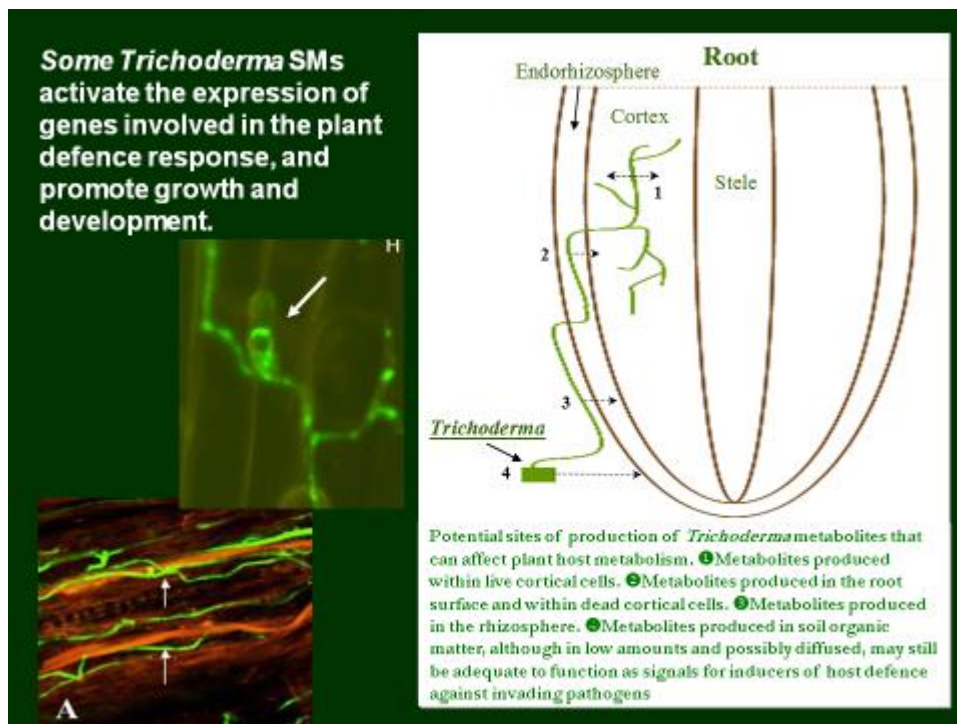
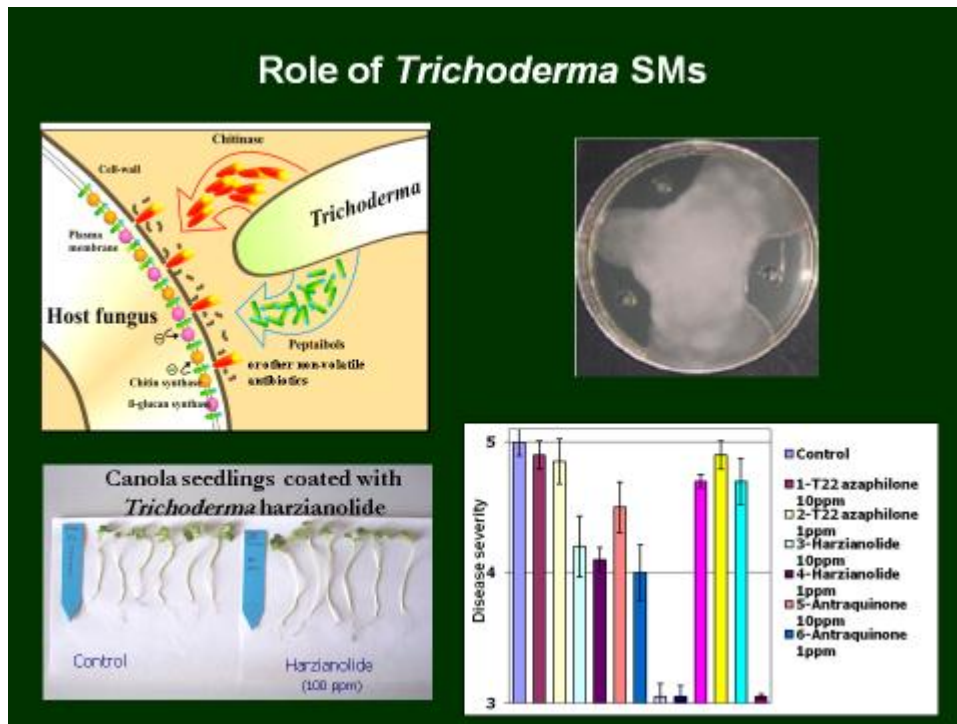
## Trichoderma secondary metabolites (SMs)

About two hundred identified :

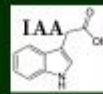
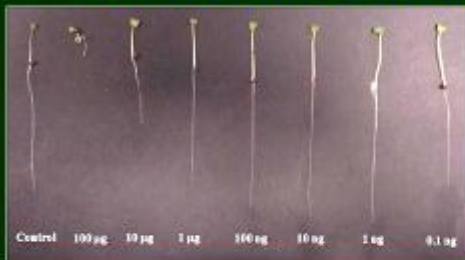
- **volatile antibiotics**, i.e. 6-pentyl- $\alpha$ -pyrone (6PP) and most of the isocyanide derivatives;
- **water-soluble compounds**, i.e. heptelidic acid or koningic acid;
- **peptaibols**, linear oligopeptides of 12-22 amino acids rich in  $\alpha$ -aminoisobutyric acid, N-acetylated at the N-terminus and containing an amino alcohol (Pheol or Trpol) at the C-terminus.



- 1: T22azaphilone;
- 2: T39butenolide;
- 3: harzianolide;
- 4: dehydro harzianolide;
- 5: harzianopyridone;
- 6: 6-pentyl- $\alpha$ -pyrone;
- 7: 1-hydroxy-3-methyl-anthraquinone;
- 8: 1,8-dihydroxy-3-methyl-anthraquinone;
- 9: harzianidione;
- 10: koningnin A;
- 11: heptelidic acid;
- 12: trichoviridin;
- 13: harzianic acid;
- 14: gliotoxin;
- 15: gliovirin;
- 16: viridin;
- 17: viridiol;
- 18: trichorzianines.



## Trichoderma SMs affect plant growth



indole-3-acetic acid (IAA) or auxin analogues.

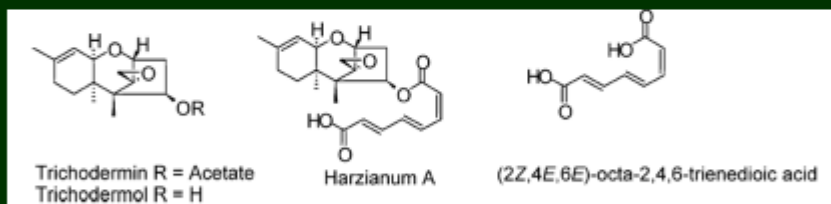
Siderophores - different families  
Produced under iron deficiency :  
coprogen (B) fusarinine C (aka fusigen) ferricrocin

- Pyrone 6-pentyl-2H-pyran-2-one (6PP)- *T. atroviride*, *T. viride*, *T. harzianum* and *T. koningii*
- koniginins A–E and G - complex pyranes - *T. koningii*, *T. harzianum*, *T. aureoviride*
- Viridiol - Steroidal antibiotic of the viridin series - *T. virens*
- Harzianopyridone, Harzianopyridone, Harzianic acid - Nitrogen heterocyclic compounds - *Trichoderma* spp.
- Cerinolactone - Lactones derivatives - *T. cerinum*
- Harzianolide and T39butenolide – Butenolides - *Trichoderma* spp.
- Trichocaranes A - D - *Trichoderma virens*
- Peptaibols

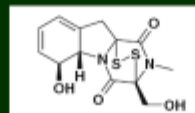
## Trichoderma toxins

Mycotoxins are secondary metabolites with adverse effects on humans and animals that result in illnesses and economic losses. Aflatoxins, ochratoxins, trichothecenes, zearelenone, fumonisins, tremorgenic toxins, and ergot alkaloids are the mycotoxins of greatest agro-economic importance.

Mycotoxins reported from *Trichoderma* include trichothecenes and gliotoxin.



Harzianum A made by esterification of trichodermol with octa-2Z,4E,6E-trienedioic acid



Gliotoxin  
(from *T. virens*)

## Trichoderma toxins

.... *T. brevicompactum* consistently produced trichodermin and/or harzianum A..... The results have important safety implications for agricultural industries because *T. harzianum*, which is widely used for biocontrol purposes, does not produce trichothecenes.....

Nielsen et al., 2005

Table 2. Trichothecene Production from Selected *Trichoderma* Strains on Semisolid and Shaken Media, Incubated for 10 Days in Darkness

medium	strain number	species	YES <sup>a</sup>		PDA		OA		PDIq		YESIq		RTIq	
			trc	harz A	trc	harz A	trc	harz A	trc	harz A	trc	harz A	trc	harz A
	IBT 40841	<i>T. brevicompactum</i>	++ <sup>b</sup>	ND	+++	ND	++	ND	++++	ND	+++	ND	+++	ND
	IBT 40840	<i>T. brevicompactum</i>	++	ND	+++	ND	++	ND	++++	ND	+++	ND	+++	ND
	IBT 40839	<i>T. brevicompactum</i>	++	ND	+++	ND	++	ND	++++	ND	+++	ND	+++	ND
	IBT 40838	<i>T. brevicompactum</i>	+	ND	+++	ND	++	ND	++++	ND	+++	ND	+++	ND
	LEO ND6	<i>T. brevicompactum</i>	++	ND	+++	ND	++	ND	not performed	not performed	not performed	not performed	not performed	not performed
	IBT 40837	<i>T. brevicompactum</i>	+	+++	+	+++	ND	+++	ND	++++	ND	++++	ND	+++
	IBT 40836	<i>T. brevicompactum</i>	ND	++++	ND	++++	+	+++	ND	++++	ND	++++	ND	+++
	IBT 40842	<i>T. brevicompactum</i>	ND	+++	+	++++	ND	+++	ND	++++	ND	++++	ND	+++
	ATCC 90237	<i>T. brevicompactum</i>	+	+++	+	+++	+	+++	ND	++++	ND	++++	ND	+++
	IBT8896	<i>T. vindex</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	IBT8212	<i>T. vindex</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	IBT8650	<i>T. harzianum</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	IBT9153	<i>T. harzianum</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	IBT9134	<i>T. harzianum</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	IBT8965	<i>T. atroviride</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	IBT8964	<i>T. atroviride</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	IBT9155	<i>T. longibrachiatum</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	IBT9128	<i>T. longibrachiatum</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	IBT9125	<i>T. citrinoviride</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	IBT9149	<i>T. citrinoviride</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

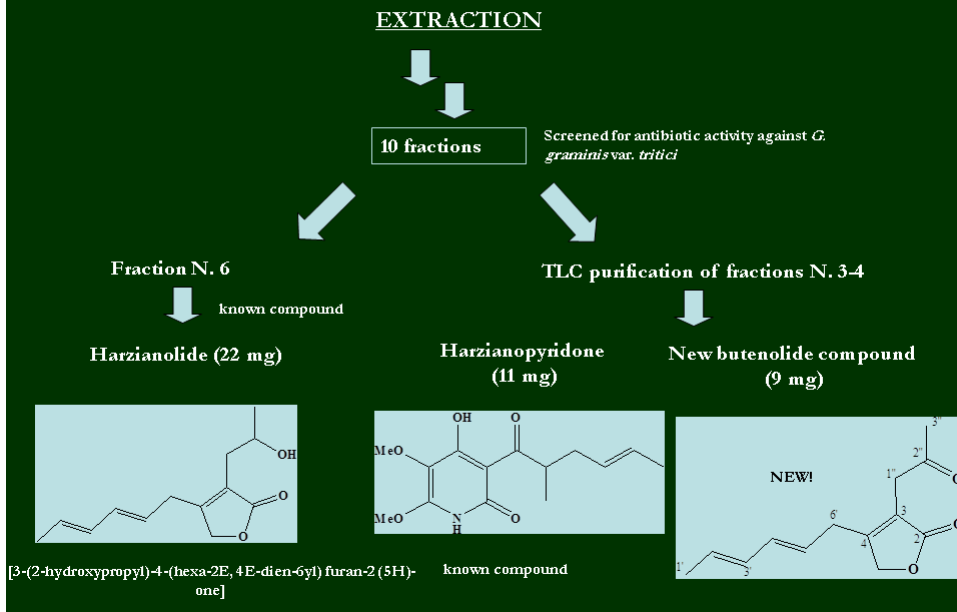
\*ND, not detected; YES, yeast extract sucrose agar; OA, oat meal agar; PDA, potato dextrose agar; YESIq, shaken yeast extract sucrose medium; PDIq, shaken potato dextrose medium; RTIq, shaken Raoult-Thom medium. <sup>a</sup>+, peak area of 5–100 counts; ++, peak area of 100–500 counts; +++, peak area of 500–3000 counts; +++++, peak area of 3000–30 000. The trichodermin peak area was determined from the m/z 263.16 (ESI<sup>+</sup>) and harzianum A from m/z 389.16 (ESI<sup>+</sup>).

## Production of Trichoderma SMs

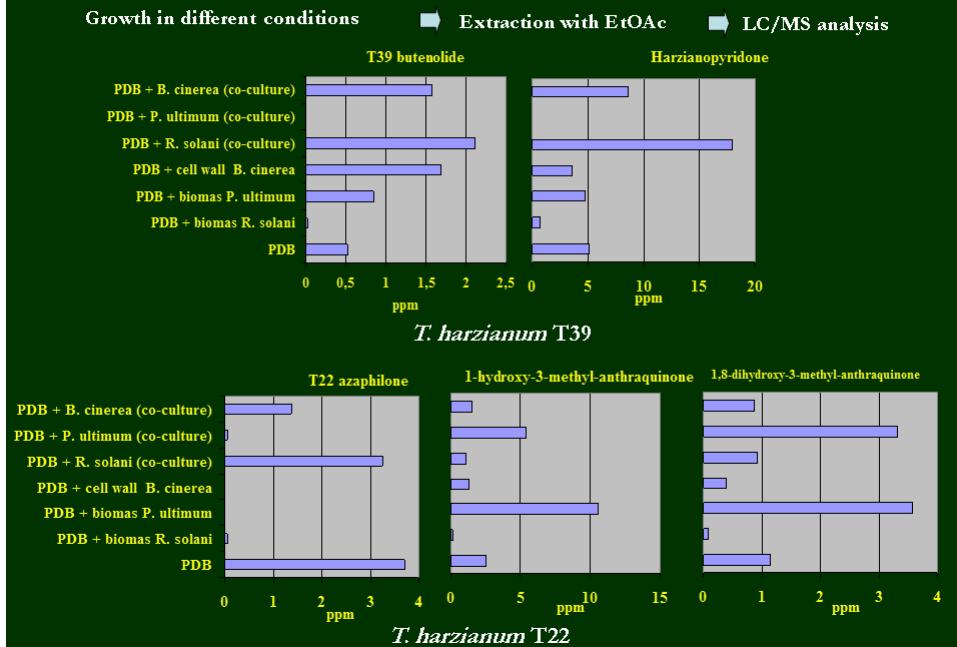
The quality and the quantity of SMs produced by *Trichoderma* depends on:

- The compound considered
- The species and the strain
- The presence of other microbes
- the balance between elicited biosynthesis and biotransformation rate
- The growth condition

The most used *Trichoderma* strains produce only a few main SMs (i.e. *T. harzianum* T39)



Production of *Trichoderma* SMs in vitro depends on the substrate



## Production of *Trichoderma* SMs in vitro in the presence and the absence of the host

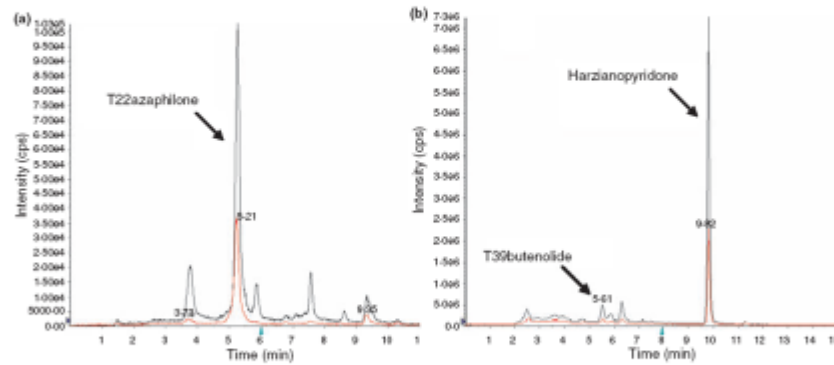


Figure 3 (a) LC/MS of extracts obtained from control (T22 alone – black) and co-culture of *Trichoderma harzianum* T22 and *Botrytis cinerea* (red); (b) LC/MS of extracts obtained from control (T39 alone – red) and co-cultures of *T. harzianum* T39 and *Rhizoctonia solani* (black).

## Production of *Trichoderma* SMs during antagonism in vitro

*T. harzianum* T22



Confrontation assay in 1/5x PDA



Plates extraction with EtOAc



TLC analysis

Results confirmed by LC/MS

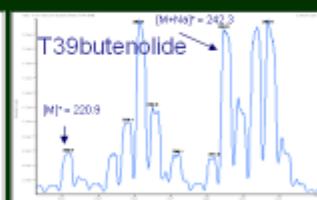
Detection of T22azaphilone



*T. harzianum* T39



Detection of T39butenolide and harzianopyridone but not the harzianolide



**Some safety issues raised relatively to inclusion  
of *Trichoderma harzianum* in Annex I**



**Full characterization of secondary metabolites  
production for each strain**

Characterization of metabolites/toxins production should be performed for each strain (and families) as it is expected that different strains would produce different metabolites. This is particularly true for trichothecenes. This has to be done in order to confirm if the mycotoxin should be taken into account in the assessment (especially for exposure via food products)

Which metabolites, eventually relevant to human and animal health, would be present in the formulation?

### **Potential translocation of secondary metabolites to the plant and the food**

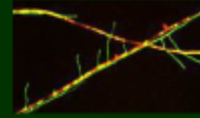
Harmful SMs would be produced in relevant quantity on the treated plants? Cannot be considered unlikely only because no information is available.

For instance, in the case of T22 the issue may concern peptaibols, (which could enter the cell by using peptide transporters) and to azaphilone or anthraquinones shown to be active on the plant.

### ***Trichoderma* SMs metabolites in the field**

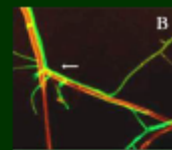
- Quick degradation of peptides in the soil and their incapacity to enter the plant are only hypothesis not supported by scientific data
- The conditions of the tests presented for toxicological data need to cover the real exposition in the field

## Some answers



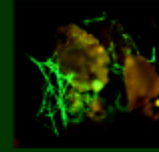
- Trichoderma BCAs have been used for many decades world-wide, no significant SMs-related problems reported so far.
- Even though many SMs are known, elite strains usually produce only a few main SMs.
- New strains properly characterized at secondary metabolite levels, and some species eventually avoided
- Production technologies that minimize the accumulation of SMs in products are available

## Some answers



- The translocation of *Trichoderma* molecules to the plant occurs, although is more appropriate to say that plant cells are just “exposed” to *Trichoderma* SMs
- Concentrations are very low and the production is limited to the interaction zone with systemic effects (no systemic translocation)
- Major mycotoxins are also toxic for the plant cell, as well as for the fungus in the culture.

### Some answers



- The production of potentially harmful compounds is normally limited in situ, and at very low level
- At least in one case dangerous secondary metabolites have been shown to be quickly degraded in the soil, but LC-MS - based evidence is needed
- The risk of exposure should be considered minimal or null. Natural soils may present high level of *Trichoderma* !

### Main issues still to be addressed

- Some main *Trichoderma* SMs may need additional toxicological studies
- Basic SM characterization of the strains in terms of SMs production in standardized conditions
- A conclusive demonstration of the absence of dangerous accumulation of the main *Trichoderma* SMs in vivo in the soil and on the plant/fruit
- Demonstrated (controlled) absence of potentially harmful content of main SMs in registered formulations
- Do not incubate product in the farm



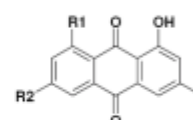
## Main groups of Trichoderma Secondary Metabolites

### *Trichoderma* secondary metabolites

#### Anthraquinones

Anthraquinones are well-known metabolites of *Trichoderma* species.  
The biological activity of anthraquinones is typically related to pigmentation

Pachybasin (1), chrysophanol (2) and emodin (3)

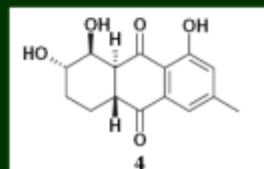


1  $R_1 = R_2 = H$

2  $R_1 = OH, R_2 = H$

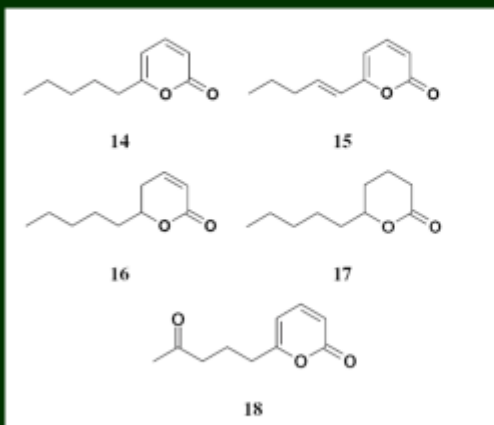
3  $R_1 = R_2 = OH$

Trichodermaol (4) is an anthraquinone-derivative isolated from the combined culture of *Trichoderma* and *Fusarium acyphorum*



## Trichoderma secondary metabolites

### Pyrones



The pyrone 6-pentyl-2H-pyran-2-one (14 or 6PP) is the representative metabolite common to the *Trichoderma* genus. This compound is a flavoring agent responsible for the coconut aroma associated with this fungus.

A strong relationship was found between the production of 6PP and the antagonistic ability of *Trichoderma*

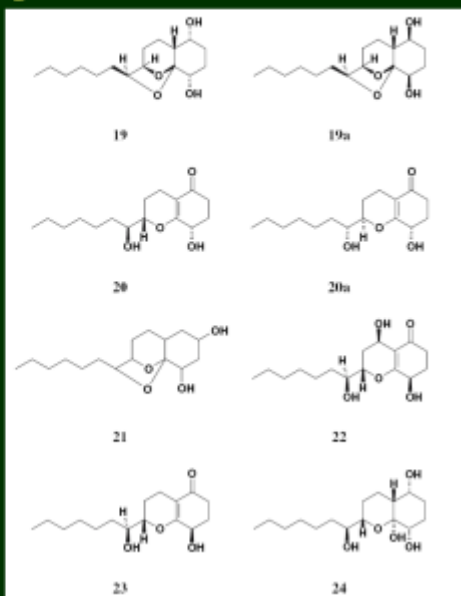
Four analogues of 6PP (15–18) have been isolated from *Trichoderma* species. From = *T. atroviride*, *T. viride*, *T. harzianum* and *T. koningii*

## Trichoderma secondary metabolites

### Koninginins

A series of complex pyranes named koninginins A–E (19–23) and G (24) were discovered in some species of *Trichoderma* (*T. koningii*, *T. harzianum*, *T. aureoviride*). Concentration-dependent activity - plant growth regulator in a wheat coleoptile assay (phytotoxic activity detected at  $10^{-3}$  M, but not at  $10^{-4}$  M).

19, 20, 22–24: antibiotic activity (i.e. towards the take-all fungus *Gaeumannomyces graminis* var. *tritici*)



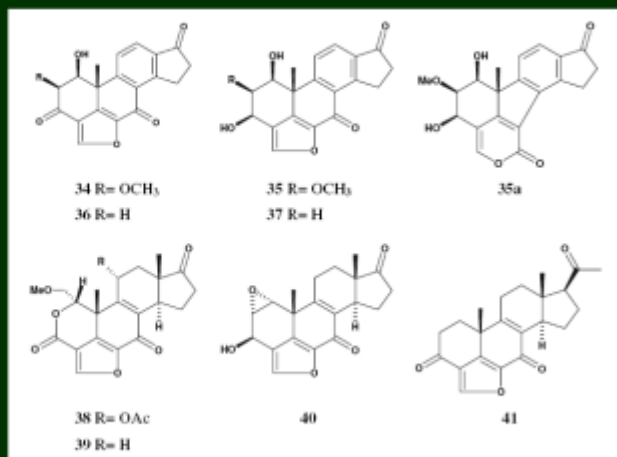
## Trichoderma secondary metabolites

### Viridins

Steroidal antibiotics of the viridin series (34–41)  
Selective antifungal activity and specific inhibitory action at specific steps in the cell signaling process.

From = *T. virens*, *T. koningii*, *T. viride*

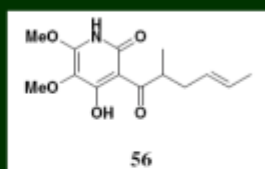
On substrates with high C/N ratios *T. virens* produce viridiol (35) that act as plant growth regulators. This compound is also an inhibitor of 5'-hydroxyaverantin dehydrogenase, an enzyme involved in aflatoxin biosynthesis.



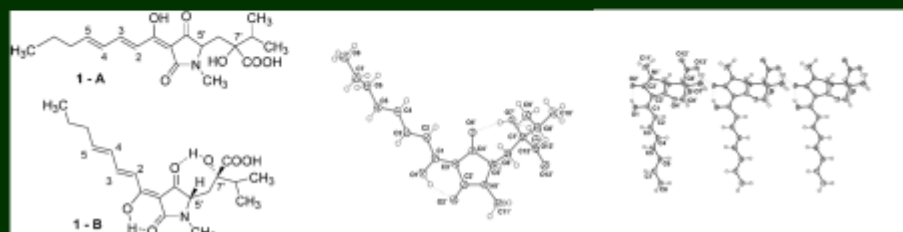
## Trichoderma secondary metabolites

### Nitrogen heterocyclic compounds

The penta-substituted pyridine ring system with a 2,3-dimethoxy-4-pyridinol pattern is present in the antifungal metabolite Harzianopyridone (56). (-)-Harzianopyridone exhibits plant growth regulating properties.



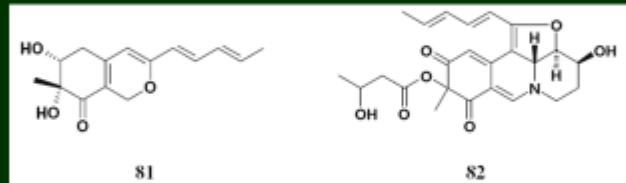
The pyrrolidindione ring system also appears in the *Trichoderma* metabolite Harzianic acid (1-B) = Antifungal and plant growth promoting metabolite



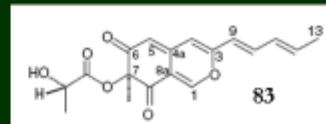
## Trichoderma secondary metabolites

### Azaphilones

Harziphilon (81) and fleophilone (82) isolated from *T. harzianum*  
 Inhibitory activity against the binding of REV-proteins to RRE RNA. 81 demonstrated cytotoxicity against the murine tumor cell line M-109



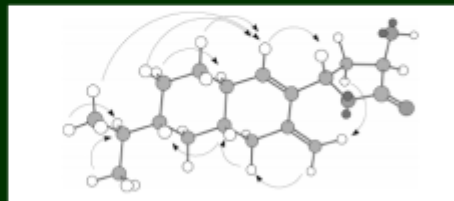
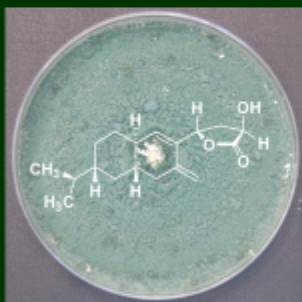
T22azaphilone (83): antifungal metabolite from *T. harzianum*



## Trichoderma secondary metabolites

### Lactones derivatives

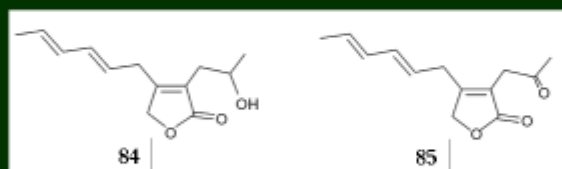
Cerinolactone from *T. cerinum* (a BCA isolated in Spain)  
 Antifungal and plant growth regulator



## *Trichoderma* secondary metabolites

### Butenolides

Harzianolide (84) and T39butenolide (85)  
Antifungal and plant growth regulator

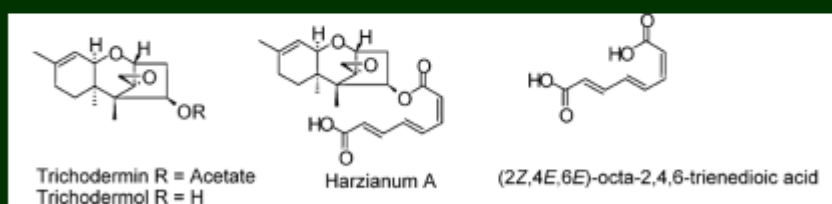


## *Trichoderma* secondary metabolites

### Trichothecenes

Mycotoxins reported from *Trichoderma* include trichothecenes and gliotoxin.  
Trichothecenes from: *T. viride*, *T. polysporum*, *T. sporulosum*, *T. reesei*

Trichodermin is inhibitor of the elongation and termination steps in the protein synthesis. Cytotoxic activity.



Harzianum A is synthesized directly by esterification of trichodemol with octa-2Z,4E,6E-trienedioic acid

## *Trichoderma* secondary metabolites

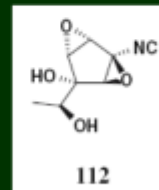
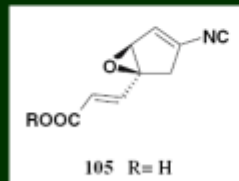
### Isocyano metabolites

Dermadin (105) = its antibiotic activity was patented in 1971

From: *T. koningii*, *T. viride*, *T. hamatum*

Trichoviridin (112) = antibiotic properties of this compound and its isolation from *T. viride* were the subject of a patent in 1970

Also from: *T. koningii*, *T. hamatum*, *T. harzianum*

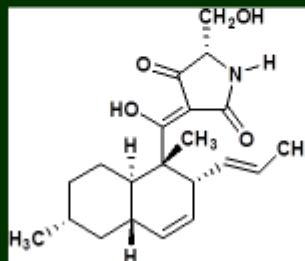


## *Trichoderma* secondary metabolites

### Setin-like metabolites

The dual culture of *T. harzianum* and calli of *Catharathus roseus* produced an antimicrobial compound named trichosedin.

This compound however, was not produced in the individual culture of *T. harzianum* or *C. roseus* callus and seems to be produced by the fungus.

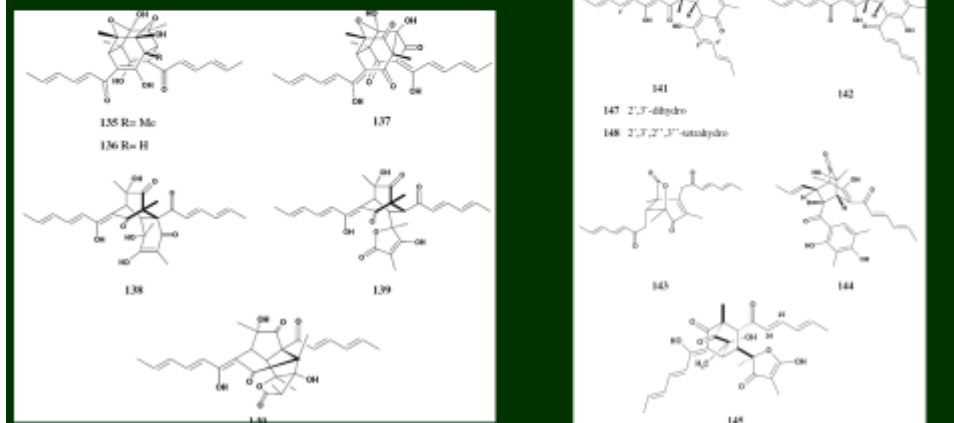
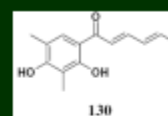


## Trichoderma secondary metabolites

### Bisorbicillinoids

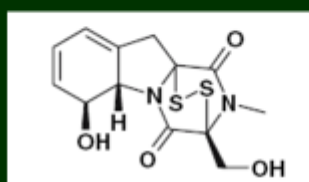
The bisorbicillinoids (i.e. Trichodimerol - 135) are a growing family of novel natural products (135-145) with interesting and diverse biological activities (antitumor, antioxidant, antibiotics, etc.) . Bisorbicillinoids are thought to be derived from sorbicillin (130) or a closely related derivative.

From: *T. longibrachiatum*, *T. citrinoviride*



## Trichoderma secondary metabolites

### Diketopiperazines



Gliotoxin  
(from *T. virens*)

Gliotoxin displays a wide range of biological effects including antiviral, antibacterial and immunosuppressive properties

## Trichoderma secondary metabolites

### Peptaibols

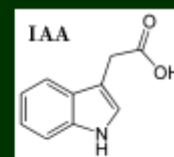
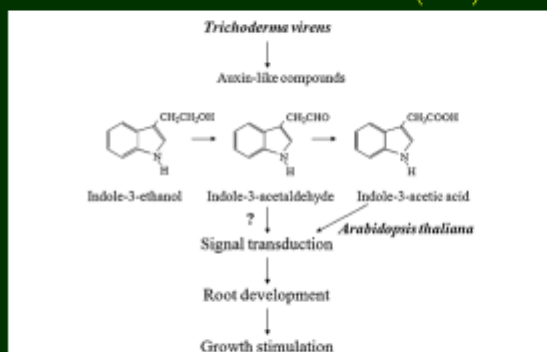
Fungal species of the *Trichoderma* genus are the main producers of this class of compounds which contain 7–20 amino acids and characteristically have an acylated N-terminal group, a C-terminal amino alcohol, and a high content of 2-amino-isobutyric acid (Aib). Unusual amino and imino acids found in peptaibols include isovaline,  $\beta$ -alanine, hydroxyproline and pipercolic acid.

### Peptabonomic

<i>T. viride</i>	
Suzukacillin A	Krause et al. (2006)
Trichotoxin A40	Breuckner et al. (1985)
Trichovirins II	Jaworski et al. (1999)
Trichovirins	Fujita et al. (1994)
Trichodecenins I, II	Fujita et al. (1994)
Trichocellins	Fujita et al. (1994)
<i>T. polysporum</i>	
Polysporins A–D	New et al. (1996)
Trichosporin B–V	Iida et al. (1993)
<i>T. reesei</i>	
Paracelsin	Breuckner et al. (1984)
<i>T. saturnisporum</i>	
Paracelsin E	Ritieri et al. (1995)
Saturnisporins SA II, SA IV	Rebuffat et al. (1993)
<i>T. koningii</i>	
Trichokonins V–VIII	Huang et al. (1995b)
Trikonigin KA, KB	Auvin-Guette et al. (1993)
<i>T. longibrachiatum</i>	
Trichobrachs A I–IV, B I–IV	Mohamed-Benkada et al. (2006)
Tricholongins B I, B II	Rebuffat et al. (1991)
Longibrachs LGBII, LGBIII	Lecterc et al. (2001)
Trichogin A IV	Auvin-Guette et al. (1992)
<i>T. harzianum</i>	
Trichorzianines A, B	El Hajji et al. (1987), Rebuffat et al. (1989)
Trichokindins I–VII	Iida et al. (1994)
Harzianins HC	Rebuffat et al. (1995)
Trichorozins I–IV	Iida et al. (1995)
<i>T. atroviride</i>	
Atroviridins A–C	Oh et al. (2000)
<i>Trichoderma</i> sp.	
Trichofumin A–D	Berg et al. (2003)

## Trichoderma plant growth regulators

Some *Trichoderma* strains enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism and are able to produce indole-3-acetic acid (IAA) or auxin analogues.

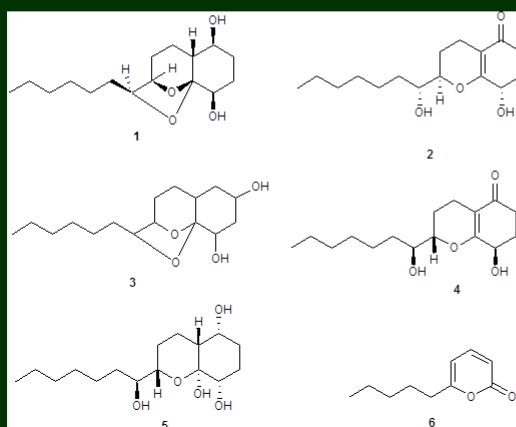


**Figure 13.** *Arabidopsis* growth responses to *T. virens* and their regulation. *T. virens* induces lateral root proliferation and enhances biomass accumulation by production of IAA and IAAld. IAAld can be converted to IAA by plant enzymes or can directly regulate auxin-inducible gene expression, possibly by interacting with auxin receptors. IET did not show clear auxin-like activity, but it can act as a storage form for other active indolic compounds such as IAAld or IAA.

## Trichoderma plant growth regulators

Secondary metabolites produced by *T. koningii* (koninginins A-C, E, G) and *T. harzianum* (6-pentyl- $\alpha$ -pyrone – 6PP) that acted as plant growth regulators.

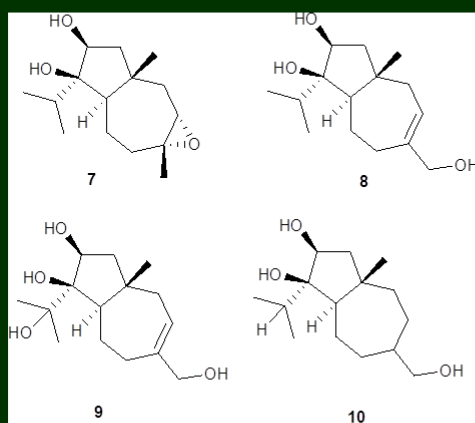
Concentration-dependent activity - plant growth regulator in a wheat coleoptile assay (phytotoxic activity detected at  $10^{-3}$  M, but not at  $10^{-4}$  M).



koninginin A, 1;  
koninginin B, 2;  
koninginin C, 3;  
koninginin E, 4;  
koninginin G, 5;  
6-pentyl- $\alpha$ -pyrone, 6.

## Trichoderma plant growth regulators

Trichocaranes A - D isolated from *Trichoderma virens* significantly inhibited the growth of etiolated wheat coleoptiles at  $10^{-4}$  and  $10^{-3}$  M

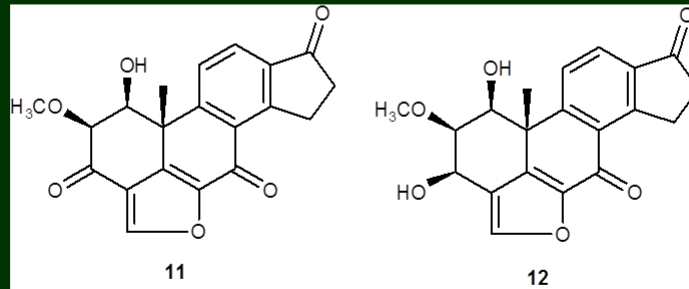


Trichocarane A, 7;  
Trichocarane B, 8;  
Trichocarane C, 9;  
Trichocarane D, 10.

## Trichoderma plant growth regulators

On substrates with high C/N ratios *T. virens* produce a metabolite similar to the antibiotic viridin called viridiol that act as plant growth regulators.

This compound is also an inhibitor of 5'-hydroxyaverantin dehydrogenase, an enzyme involved in aflatoxin biosynthesis.



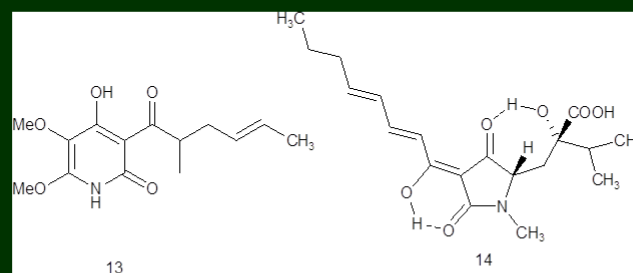
Viridin, 11; Viridiol, 12

## Trichoderma plant growth regulators

(-)-Harzianopyridone exhibits plant growth regulating properties.

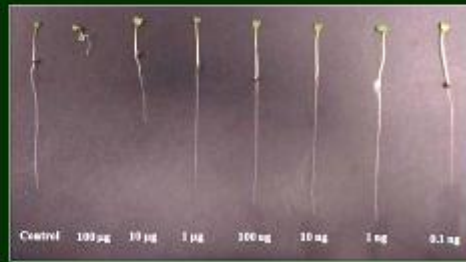
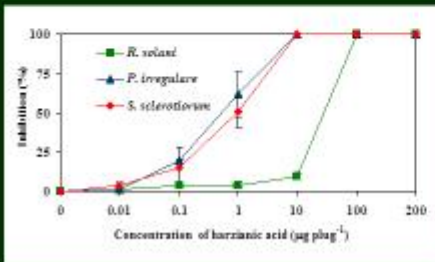
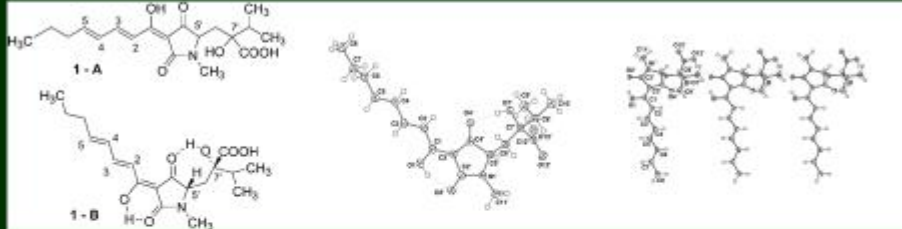
Harzianic acid has been purified from the culture filtrate of a strain of *Trichoderma harzianum* isolated from composted hardwood bark.

Growth of canola seedlings was affected by treatments with harzianic acid.



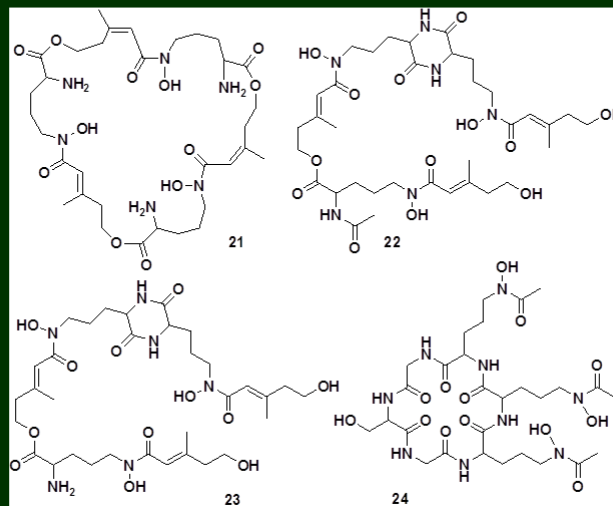
Harzianopyridone n. 13;  
Harzianic acid n. 14.

## *Harzianic acid* *Antifungal and plant growth promoting metabolite*



## *Trichoderma siderophores*

Different structural families from culture filtrate of *Trichoderma* spp.  
Under condition of iron deficiency the culture filtrate contained coprogen (21),  
coprogen B (22), fusarinine C (23, also called fusigen) and ferricrocin (24).



**Fate and behaviour of *Trichoderma* in the soil and effects on soil microflora  
[PPT 5]**

*Ilaria Pertot (Fondazione Edmund Mach, FEM-IASMA, Plant Protection Dept., San Michele all'Adige;  
Italy)*

## FATE AND BEHAVIOR OF TRICHODERMA IN THE SOIL AND EFFECTS ON SOIL MICROFLORA

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**Ilaria Pertot**

Fondazione Edmund Mach,  
Sustainable agro-ecosystems and bioresources Dept.  
San Michele all'Adige, Italy

## ECOLOGY OF *TRICHODERMA* SPP.

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## *Trichoderma* spp. are ubiquitous fungi in soils in all climatic zones

- *Trichoderma* spp. are a fast-growing group of fungi, extremely common in agriculture, forest, grassland and desert soils
- In all climatic conditions
- Particularly prevalent in the litter of humid forests (up to 3% of the total fungal propagules)
- And pasture soils (up to 1.5%)
- Commonly 10-10<sup>3</sup> CFU/g soil
- High ecological adaptability

## Example of some *Trichoderma* spp. isolated from soils

<i>Trichoderma</i> species	Origin
<i>asperellum</i>	France, <i>Beta vulgaris</i> , soil
<i>viride</i>	England, <i>Pinus</i> sp.
<i>hammetum</i>	India, livestock food
<i>atroviride</i>	France, soil
<i>harzianum</i>	Colombia, soil
<i>harzianum</i>	Philippines, soil
<i>longibrachiatum</i>	Spain, air
<i>saturisporum</i>	Australia, <i>Triticum</i> sp., rhizosphere
<i>harzianum</i>	Spain, soil
<i>asperellum</i>	Colombia, soil
<i>koningi</i>	England, <i>Lycopersicon esculentum</i> , soil
<i>atroviride</i>	England, soil
<i>harzianum</i>	USA, soil
<i>asperellum</i>	Spain, compost
<i>aggressivum</i> f. <i>europaeum</i>	Spain, compost
<i>atroviride</i>	Zimbabwe, soil
<i>harzianum</i>	India, soil
<i>aureoviride</i>	Colombia, soil
<i>harzianum</i>	England, soil
<i>harzianum</i>	England, soil
<i>harzianum</i>	England, soil
<i>harzianum</i>	England, soil
<i>harzianum</i>	India, <i>B. vulgaris</i> , soil
<i>harzianum</i>	India, <i>L. esculentum</i> , soil
<i>longibrachiatum</i>	India, <i>B. vulgaris</i> , soil
<i>harzianum</i>	Sri Lanka, soil
<i>asperellum</i>	Spain, <i>B. vulgaris</i> , soil

From Rubio et al., 2005. *Microbial ecology* 49: 25–33

### *Trichoderma* spp. can metabolize a variety of substrates

- **Saprophytes**, they can parasitize other fungi
- Tolerate a **wide range of pH levels**
- Can utilize a **large number of carbon and nitrogen sources**
- **Good cellulolytic activity**
- However **low ability to degrade wood**
- They colonize wood by **degrading non-structural carbohydrates**
- Use as wood preservatives: they consume sugars and starch and limit the attack by secondary invaders (wood rots) and direct attack towards wood decay fungi

### *Trichoderma* spp. can adapt to a wide range of environmental conditions

- They grow well at temperatures between **10 and 30 °C**
- **Optimal between 25 to 30°C, high humidity**
- Some correlation between **species distribution and environmental conditions** (es. *T. harzianum* more prevalent in warmer climates)
- **Relatively low concentration in drier forest litter layer**
- Generally **more resistant to chemical treatment to the soil** (dominant after fumigations)
- **Resistant to relatively high temperature** (dominant after solarization)

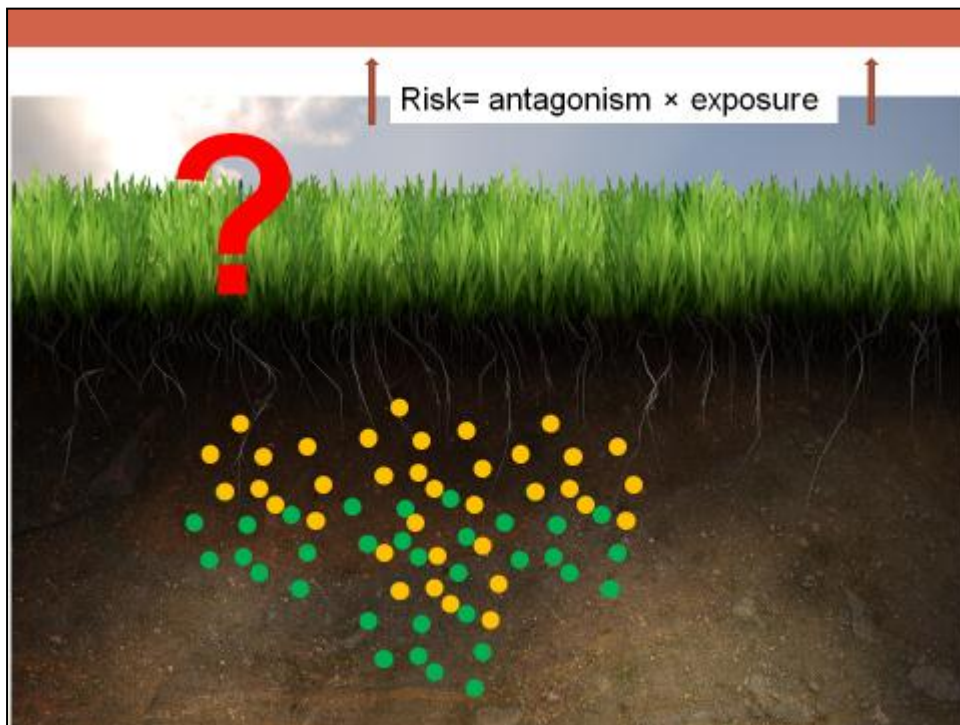
## Mechanisms of action of *Trichoderma* spp.

- **Mycoparasitism:** a species or strain of fungus directly attacks and feeds on other fungi
- **Antibiosis:** production of antibiotics or enzymes that inhibit the growth or reduce the competitive ability of other organisms
- **Competition for space and resources:** quick colonization, creating an effective 'living barrier' to subsequent pathogen invasion
- **Induced resistance:** changes in the plant that increase disease resistance against diseases



Fig. 1. Penetration and haustoria formation within the large hyphae of *Rhizoctonia solani* by the smaller hyphae of *Trichoderma virens*.

From Howell, 2003. Plant Disease, 87 4-10



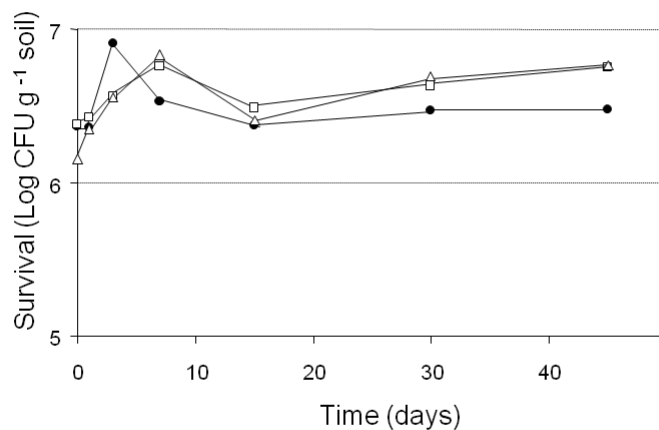
## FATE AND BEHAVIOR OF *TRICHODERMA* SPP. IN THE SOIL

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## The introduced *Trichoderma* strains usually decrease over time

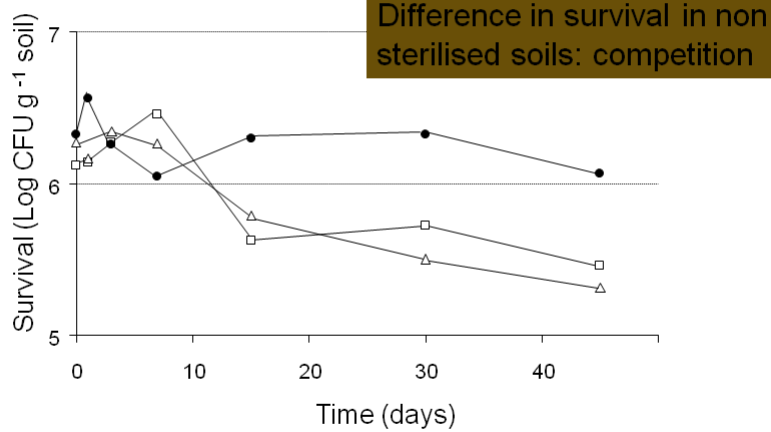
- Without suitable substrates the introduced *Trichoderma* strain **fast decreases** to the level of the native *Trichoderma* spp. or lower (competition with existing microflora)
- A major challenge is to **maintain stable populations** so that beneficial effects are sustained throughout the growing season
- **Compost** may increase the survival of the introduced *Trichoderma* strain in soil
- **Soil fumigation** can increase the presence of indigenous *Trichoderma* spp. on roots
- Several strains: **good rhizosphere competence**

### *T. atroviride* SC1 – sterile soil microcosms

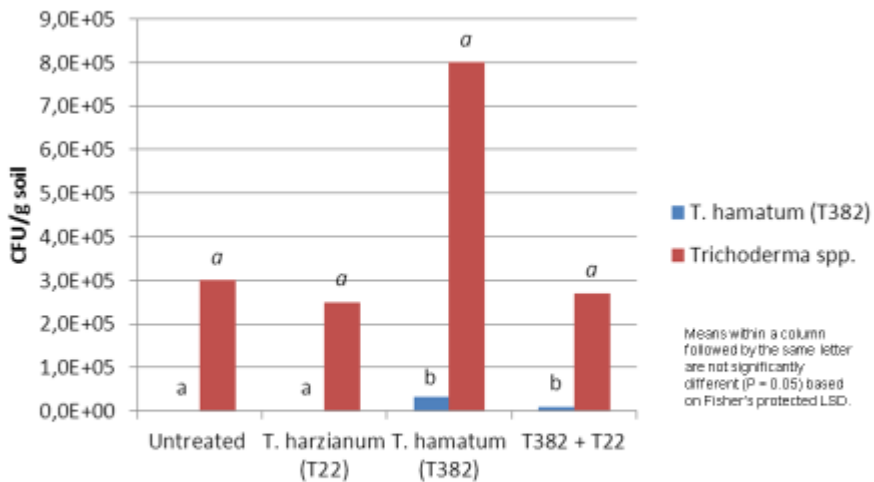


3 sterilized soils were inoculated at day 0 by a conidia-water suspension ( $1.5 \times 10^6$  CFU·ml<sup>-1</sup>) with no formulation

### *T. atroviride* SC1 – non sterile soil microcosms

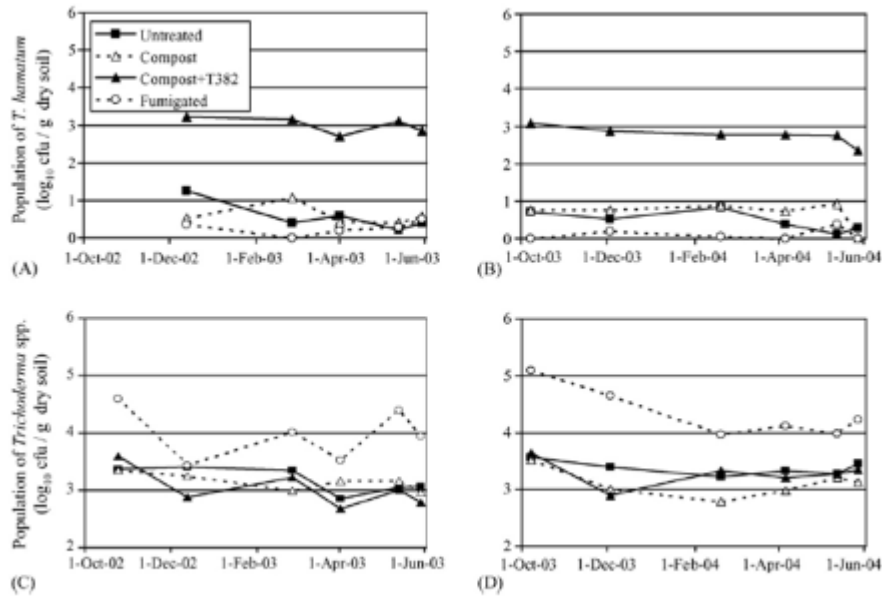


3 non sterilized soils were inoculated at day 0 by a conidia-water suspension ( $1.5 \times 10^6$  CFU·ml<sup>-1</sup>) with no formulation



Populations of *Trichoderma hamatum* (T382), *T. harzianum* (T22) and *Trichoderma* spp. in potting mixes used to grow strawberry plug transplants for 4 weeks in the greenhouse. Initial T382=  $2 \times 10^5$ ; T22=  $6 \times 10^4$

Modified from L.F.S. Leandro et al. / Applied Soil Ecology 35 (2007) 237–246



From L.F.S. Leandro et al. / Applied Soil Ecology 35 (2007) 237–246

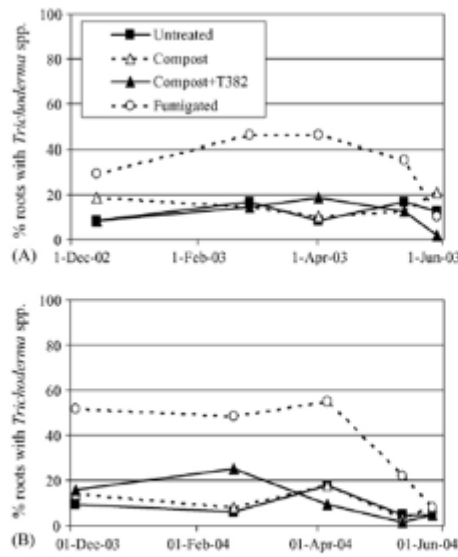
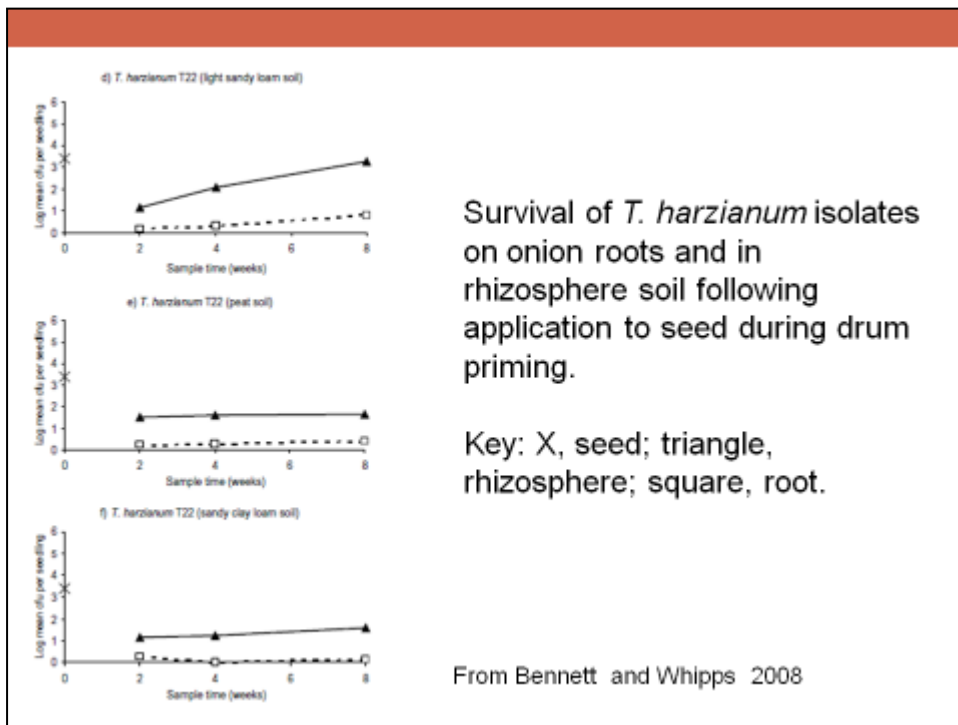


Fig. 2. Frequency of isolations (% root pieces) of indigenous *Trichoderma* spp. (all spp. except *T. hamatum*) from strawberry roots growing in different soil treatments in (A) year 1 and (B) year 2.

From L.F.S. Leandro et al. / Applied Soil Ecology 35 (2007) 237–246



## DETECTION AND QUANTIFICATION OF *TRICHODERMA* SPP. IN SOILS

## Microbiological methods

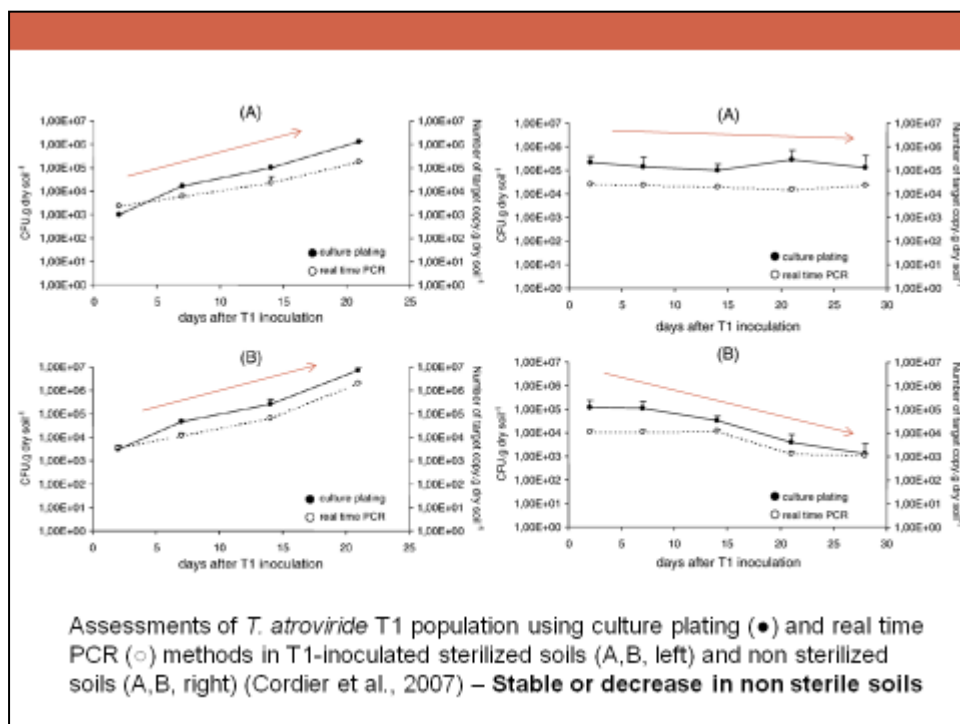
- Dilution plates on **selective or semi-selective media**
- This method **does not distinguish between indigenous strains and artificially introduced ones**
- Calculates the **colony forming unit (CFU)**, which can be either a conidium or a growing colony (mycelium)

## Molecular methods

- Staining techniques, based on **reporter genes** such as the b-glucuronidase gene (GUS); cannot be applied in environmental studies (release of GMO strains)
- Sequence analysis of the **internal transcribed spacer region (ITS)** of the ribosomal DNA and from other multiple genetic loci
- **Randomly amplified polymorphic DNA (RAPD)** technique
- These PCR-based techniques **do not allow accurate quantification** of DNA because of the variability in the efficiency of amplification between PCR reactions

## Molecular methods

- Conversion of a **RAPD derived fragment into a sequence-characterized amplified region (SCAR)**
- Realtime PCR based on SCAR marker can be used to quantify fungal DNA of a specific strain extracted from soil samples
- The amount of DNA recovered from a given soil is highly related to factors such as soil type or the method of extraction
- Initially permitted to quantify DNA from sterile soil artificially infested with  $1 \times 10^8$  fungal spores/g of soil and did not permit to detect the strain among a *Trichoderma* mixture introduced in soil (Rubio et al., 2005)
- Later was possible at levels as low as  $1 \times 10^3$  CFU/g of **non-sterile soil**, without amplification of autochthonous populations of *Trichoderma* spp. (Cordier et al., 2007, Feng et al., 2011)

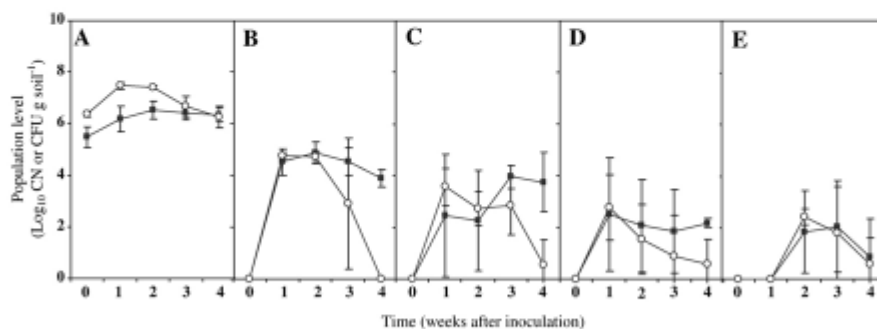


## Real-time PCR primers and strain specific TaqMan probe set

- Real-time PCR amplification of a amplicon of 110 bp of the endochitinase 42 gene (*ech42*), present in all *Trichoderma* species, and a strain-specific signal TaqMan probe based on the uniqueness of two nucleotide mutations in the sequence of the first intron of the *T. atroviride* SC1 strain
- Comparison between CN and CFU in microcosm (Savazzini et al., 2008)

## Real-time PCR primers and strain specific TaqMan probe set

number of copies of haploid genomes (CN) quantified by real-time PCR (■), and the number of colony forming units (CFU) counted on PDA plates (○) at different depths in soil



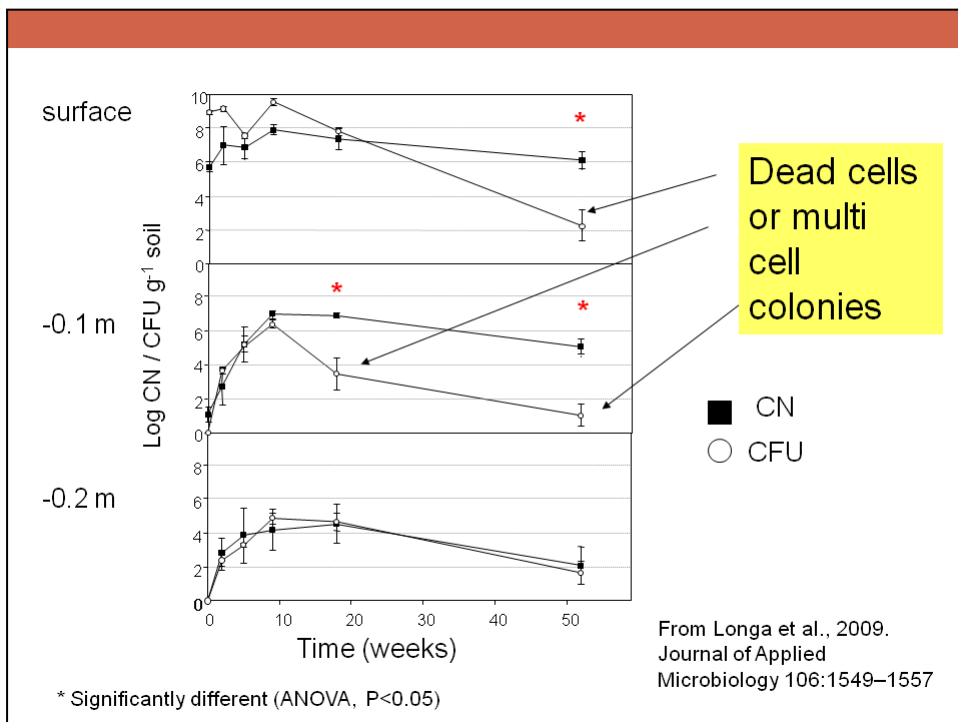
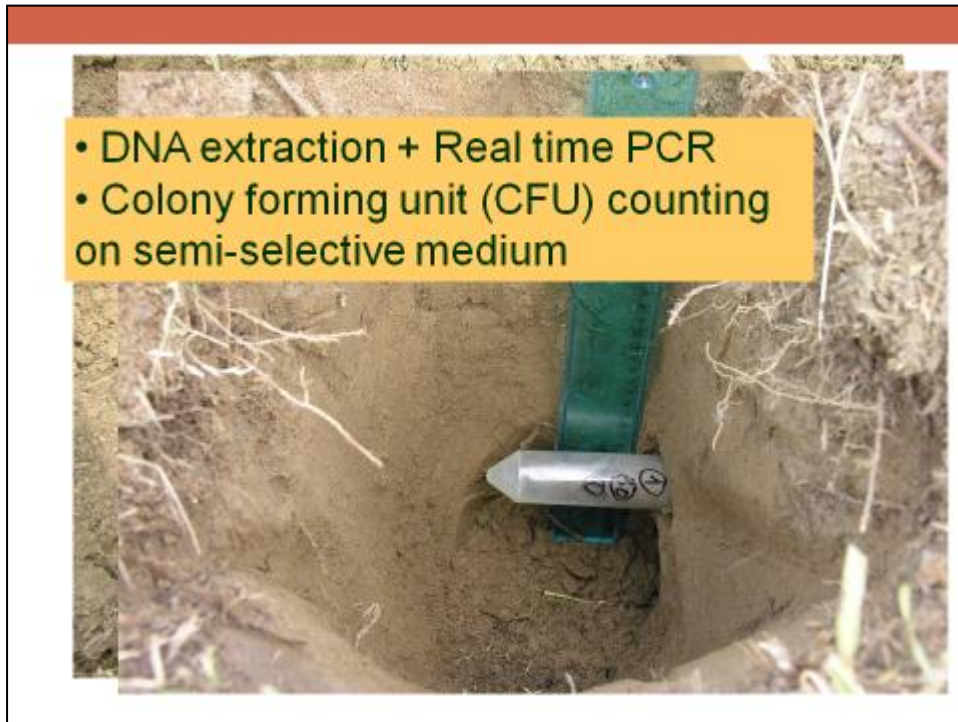
From Savazzini et al., 2008

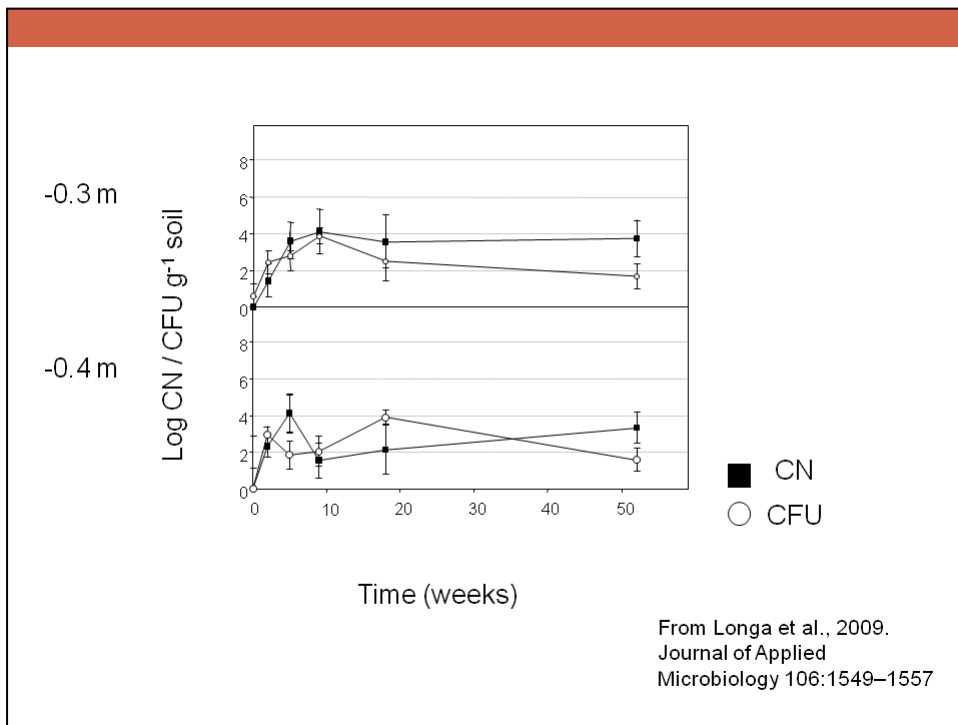
## Survival and vertical dispersion in soil (2006-2007)

- Six plots of 0.6 × 0.6 m each, located between grapevine plants in the row in a vineyard
- Three plots were inoculated with *T. atroviride* SC1 (500 g of the boiled-rice with the fungus grown on it)
- The inoculum was mixed into the soil surface layer (approximately 3 cm deep)
- The initial concentration of the fungal inoculum in this layer was estimated to be  $10^8$  CFU g dry soil<sup>-1</sup>

Longa et al., 2009. Journal of Applied Microbiology 106:1549–1557







MAY *TRICHODERMA* SPP. HAVE  
DETRIMENTAL IMPACTS ON OTHER  
MICROORGANISMS PRESENT IN  
THE ECOSYSTEM TO WHICH THEY  
ARE APPLIED?

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## Non-target effects of biological control agents

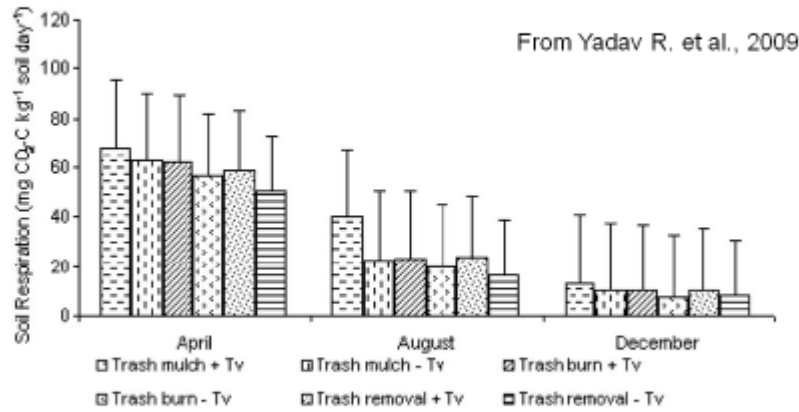
From Cook et al., 1996:

- **Competitive displacement:** competitive displacement occurs when a BCA expels or replaces native non-target species through competition for space or nutrients
- **Pathogenicity:** the potential to infect organisms other than the target pests
- **Allergenicity:** may occur in humans or animals that develop sensitivities to spores or formulations
- **Toxicity:** release of antibiotics or alkaloids, which may have toxic effects on non-target species

## Possible non-target effect of *Trichoderma* spp. in soils

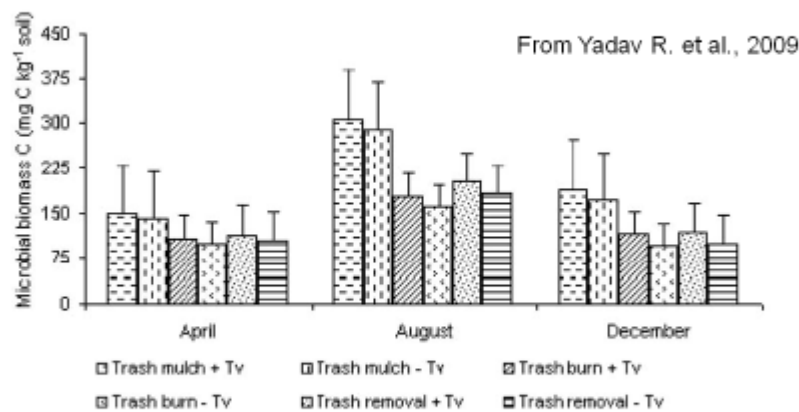
- Reduction in the **diversity and/or abundance** of other fungi in the soil
- **Disrupt entire ecosystems** through changes in the native soil community
- Ecological effects on plants that form **symbiotic relationships with fungi** (mycorrhizae)
- Affect some insects that form complex relationships with fungal species

### No major effects of *T. viride* on soils populations



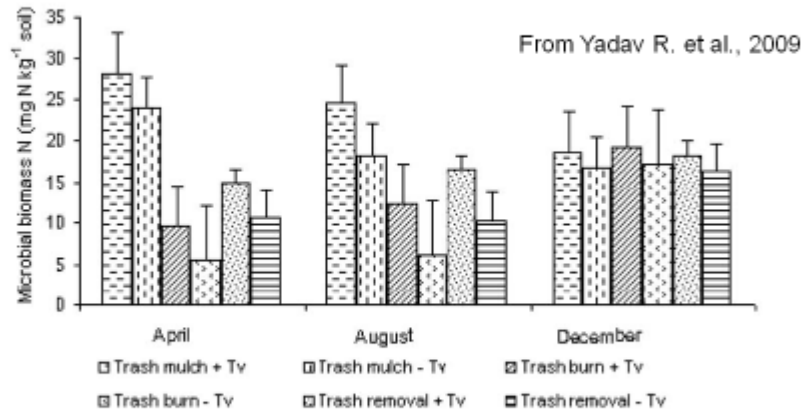
Effect of different trash (recycling stripped dry cane leaves for conserving soil organic matter) management practices in ratoon sugarcane with and without *Trichoderma* inoculation on **cumulative soil respiration after 30 days**

### No major effects of *T. viride* on soils populations



Effect of different trash (recycling stripped dry cane leaves for conserving soil organic matter) management practices in ratoon sugarcane with and without *Trichoderma* inoculation on **soil microbial biomass carbon (SMBC)**

## No major effects of *T. viride* on soils populations



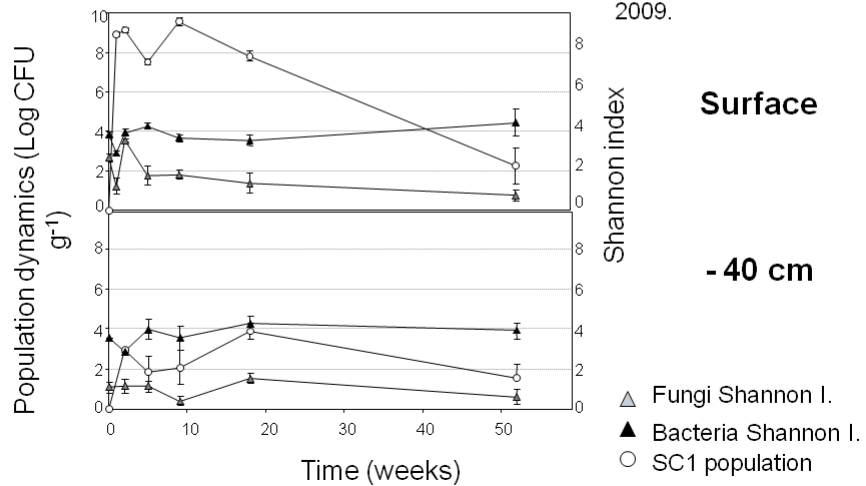
Effect of different trash (recycling stripped dry cane leaves for conserving soil organic matter) management practices in ratoon sugarcane with and without *Trichoderma* inoculation on **soil microbial biomass nitrogen (SMBN)**

## Impact of *T. atroviride* SC1 on microbial communities in soil

- Native communities of bacteria and fungi in the soil were analyzed using **automated ribosomal intergenic spacer analysis (ARISA)**
- PCR amplification of the intergenic region between the small and the large subunit rRNA genes in the rRNA operon with fluorescence-tagged oligonucleotide primers from the total bacterial or fungal community DNA and automated detection of fluorescent DNA fragments (Savazzini et al., 2009)

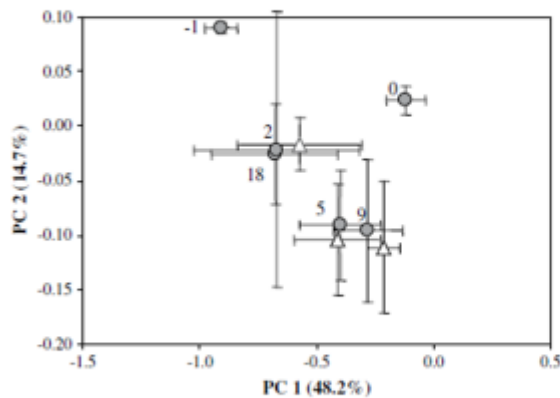
## Impact of *T. atroviride* SC1 on microbial communities in soil: biodiversity

From Savazzini et al., 2009.



## Impact of *T. atroviride* SC1 on microbial communities in soil: biodiversity

From Savazzini et al., 2009.



Effect of the *T. atroviride* SC1 inoculum on the soil bacterial community from the field experiment

**Fig. 3.** Effect of the *Trichoderma atroviride* SC1 inoculum on the soil bacterial community from the field experiment. The plot of the first two component of variance (PC 1 and 2) was generated from the ARISA profiles, as weekly averages (-1 h, 0 inoculum time, 2, 5, 9 and 18 weeks after inoculation) of the inoculated (●) and control areas (Δ). The vertical and horizontal bars represent the standard deviations (n = 5).

## Impact of *T. atroviride* SC1 on microbial communities in soil: biodiversity

From Savazzini et al., 2009.

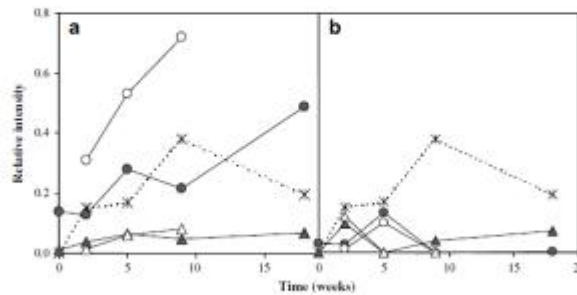
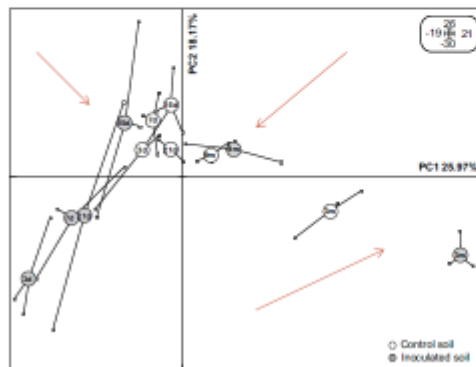


Fig. 6. Population dynamics of several of the most representative soil fungi in the surface layer of treated (black) and control (white), expressed as relative fluorescence intensity from the F-MRSA. (a) *Aspergillus* spp. (● and ○) and *Ascaris* spp. (▲ and △); (b) *Penicillium* spp. (● and ○) and *Cladosporium* spp. (▲ and △). The *T. atroviride* SC1 is shown in both figures as dotted line (x).

## *T. atroviride* on non target soil micro-organisms

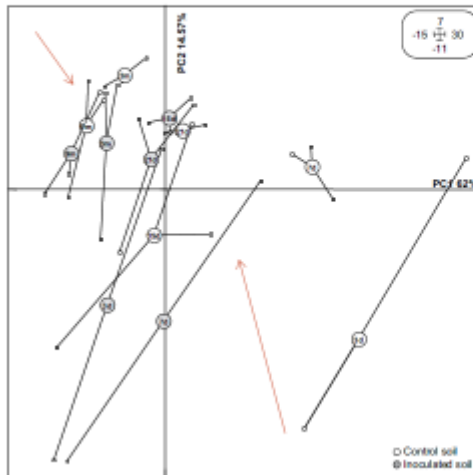
- The introduction of the strain *T. atroviride* I-1237 into two soils slightly modified the microbial diversity, only for a short period of time



Principal component analysis (PCA) of T-RFLP profiles for structure of the **fungal community in Marlins soil** 3 days to 9 months after or without inoculation of BCA strain of *T. atroviride*

From Cordier and Alabouvette, 2009

## *T. atroviride* on non target soil micro-organisms



Principal component analysis (PCA) of T-RFLP profiles for structure of the **bacterial community in Epoisses soil** 3 days to 9 months after or without inoculation of BCA strain of *T. atroviride*

From Cordier and Alabouvette, 2009

### Log total pasture soil and root bacteria and fluorescent *Pseudomonad* populations as affected by *Trichoderma* inocula and *Pythium ultimum*

	Soil without <i>Pythium</i>						soil treated with <i>Pythium</i>					
	Control	To10	TH1	T4	N47	T12	Control	To10	TH1	T4	N47	T12
R-bact	7.24a	7.13a	7.28a	7.29a	7.36a	7.27a	8.01bc	8.07bc	7.99bc	8.39d	7.85b	8.14c
S-bact	7.55c	6.91cd	7.02d	6.38a	6.73b	6.84bc	8.28g	8.15g	7.75f	8.30g	8.20g	8.44g
Pseu	5.53ab	5.30a	5.48ab	5.54b	5.48ab	5.26a	5.98cd	6.18c	5.86bc	5.98cd	5.93cd	6.11de

To10, TH1, T4, N47, T12: *Trichoderma* strains inoculated. R-bact: root bacteria  $g^{-1}$  root; s-bact soil bacteria  $g^{-1}$  soil; pseu: fluorescent pseudomonads  $g^{-1}$  root. Within the same column, values with no letters in common are significantly different at  $P < 0.05$  level.

### Log fungal populations as affected by *Trichoderma* inocula and *Pythium ultimum*

	Soil without <i>Pythium</i>						Soil treated with <i>Pythium</i>					
	Control	To10	TH1	T4	N47	T12	Control	To10	TH1	T4	N47	T12
R-fungi	5.15a	5.41c	5.25ab	5.40bc	5.35bc	6.19f	6.10e	6.12e	5.72d	6.21f	5.91de	6.17f
S-fungi	5.48b	5.36a	5.95c	6.26e	5.56b	6.25c	6.26e	6.13d	6.47f	6.34e	6.31e	6.14d
S-Trich	ND	4.41ab	4.00a	4.15a	4.45ab	4.68abc	ND	4.87bc	4.66ab	4.99c	4.87bc	4.56ab
R-Trich	ND	4.30a	4.35ab	4.30ab	4.48ab	4.26a	ND	5.18e	4.86bc	4.88cd	4.93cd	5.01de
<i>Pythium</i>	ND	ND	ND	ND	ND	ND	6.10c	5.99bc	6.10c	5.94ab	5.93ab	5.83a

To10, TH1, T4, N47, T12: *Trichoderma* strains inoculated. R-fungi: root fungi  $g^{-1}$  root; s-fungi: soil fungi  $g^{-1}$  soil; S-Trich: soil *Trichoderma/g^{-1} soil; r-Trich: root *Trichoderma/g^{-1} root; *Pythium*: *Pythium/g^{-1} soil; ND: not detected. Within the same column, values with no letters in common are significantly different at  $P < 0.05$  level.***

From Naseby et al., 2000. *Journal of Applied Microbiology* 88: 161–169

## *Trichoderma* spp. and arbuscular mycorrhizal species

Reviewed by Brimmer and Boland, 2003

- *T. harzianum* can penetrate the resting spores and subtending hyphae of *Glomus intraradices* and in the hyphae of *Glomus mosseae*
- *T. koningii* reduces germination of resting spores of *G. mosseae*, but only the resting spore stage is affected
- *T. harzianum* had no effect on hyphal biomass, sporulation or phosphorus transport activity and the external mycelium of *G. intraradices*
- *T. harzianum* or *T. aureoviride* had no detrimental effect on germination of resting spores of *G. mosseae*
- *T. harzianum* or *T. pseudokoningii* effect on the germination of resting spores or hyphal growth rate of *G. mosseae* was insignificant

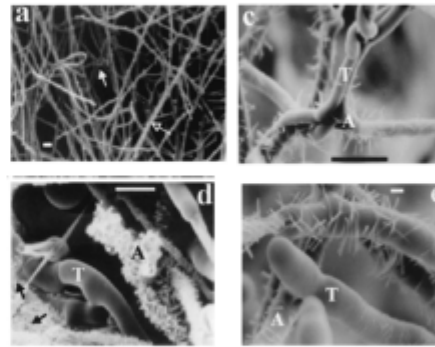
## *Trichoderma* spp. and arbuscular mycorrhizal species

Reviewed by Brimmer and Boland, 2003

- *Trichoderma* spp.: **positive effects on the growth and propagation of mycorrhizal fungi.** Inoculation of *G. mosseae* spores, with *T. harzianum* and *T. aureoviride* decreased the time to vegetative sporulation and increased the number of asexual spores produced by *G. mosseae*
- The ability of *Trichoderma* spp. to impede colonisation of plant roots by arbuscular mycorrhizal species **depends on the order of inoculation of the fungi.** Colonisation by *G. mosseae* was reduced only when plants were simultaneously inoculated with *T. koningii* and *G. mosseae* or when *T. koningii* was inoculated 2 weeks before. No effect if *G. mosseae* was inoculated 2 weeks prior to *T. koningii*
- *Trichoderma* spp. have a negligible or **even stimulatory effect on the colonisation of plant roots by mycorrhizal fungi**

## Aggressiveness of *T. harzianum* toward the Commercial Mushroom *Agaricus bisporus*

- *T. harzianum* associated with a cropping disorder in the commercial white mushroom
- The aggressiveness results from extensive saprotrophic growth involving degradation of wheat straw cell wall
- Only as a component: competition, antagonism, or parasitism



From Williams et al., 2003. App. Env. Microbiol. 69: 4192–4199

## Summarizing:

- Relatively **fast decrease** of the introduced Trichoderma strain in soil due to the **competition with existing microflora** (*general*)
- Survival on roots or rhizosphere if the Trichoderma strain is rhizosphere competent (*strain-dependent*)
- **Temporary and almost negligible** effect on natural bacterial or fungal soil microflora (*general*)
- **No effect** on symbiotic arbuscular mycorrhiza when applied after or during mycorrhization (*almost general*)
- **Specific aggressive strain** causing diseases in *A. bisporus* (*strain specific*)

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**Fate of a strain of *Trichoderma atroviride* in soil and effects on the soil microbial communities**  
**[PPT 6]**

*Claude Alabouvette (AGRENE, Dijon; France)*

**Fate of a strain of *Trichoderma atroviride* in soil and effects on soil microbial communities**

**Claude Alabouvette and Christelle Cordier**

AGRENE 47 Rue Constant Pierrot, F. 21000 DIJON



**Design of a SCAR marker**

- A SCAR (sequence amplified characterized region) marker is the best tool to address the question of fate and behaviour in the environment.
- It can be used to specifically detect but also quantify the introduced strain among the autochthonous population of the same species

BPSG-seminar: Trichoderma for the use of Plant Protection Products: similarities and differences

## **SCAR marker of the strain I-1237 of *Trichoderma atroviride***

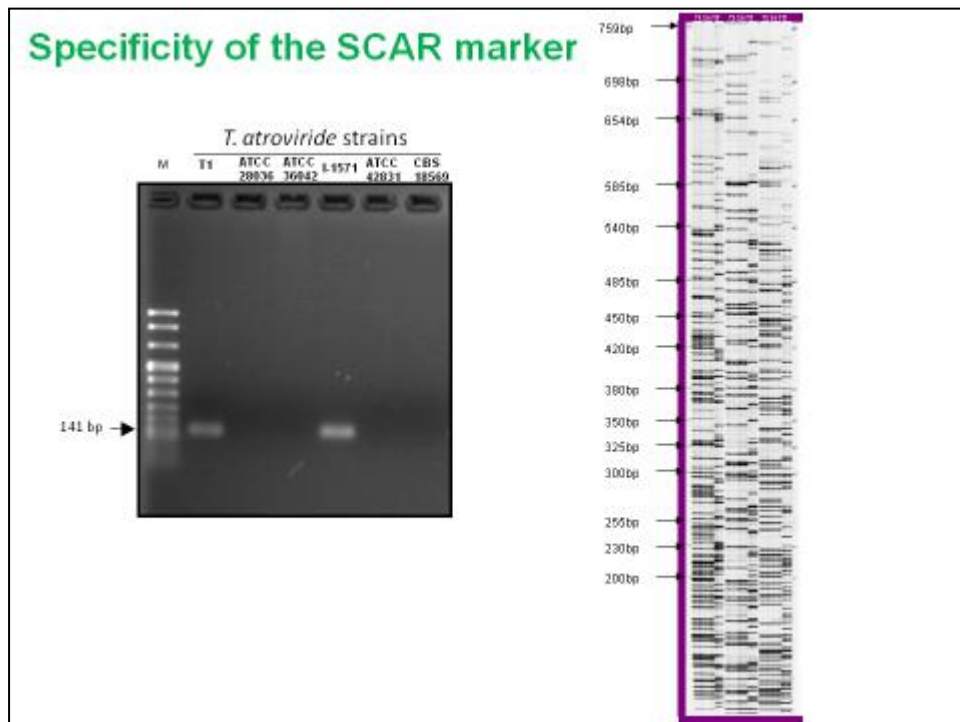
- We designed a SCAR marker to answer regulation requirements:
- 1/ tool for identification of the MBCA at the strain level
- 2/ study of the fate and behaviour in the environment
- 3/ study of residues on food and feed

BPSG-seminar: Trichoderma for the use of Plant Protection Products: similarities and differences

## **Approach**

- Looking into the genome of the strain to identify a sequence present only in that strain
- Different techniques can be used: RAPD, AFLP...
- Check for the specificity of the sequence
- Design of primers enabling to amplify a sequence compatible with the use of quantitative real time PCR

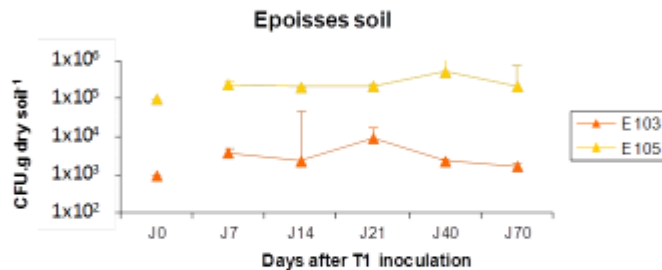
BPSG-seminar: Trichoderma for the use of Plant Protection Products: similarities and differences



## Validation of the method

- Choice of two soils of very different physico-chemical properties
- Introduction of the strain I-1237 into these soils, at two different inoculum concentrations ( $10^3$  and  $10^5$  CFU  $g^{-1}$  soil)
- Study of population kinetics by the soil dilution technique and identification of the strain thanks to the SCAR marker

**Population dynamics of strain I-1237 of *Trichoderma atroviride* in Epoisses soil**

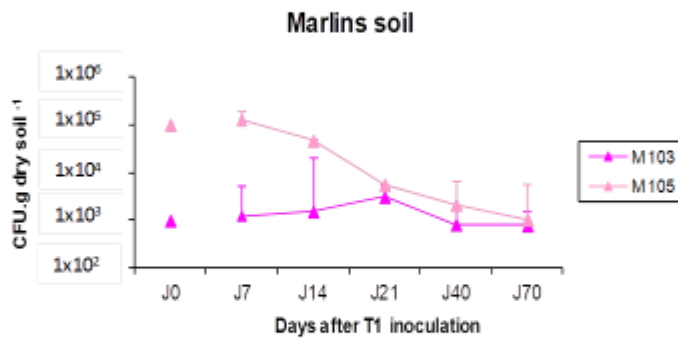


Soil dilution technique followed by identification of T1 strain by PCR using the primers designed for the SCAR

**In this soil the strain established at a level close to the initial concentration**

*BPSG-seminar: Trichoderma for the use of Plant Protection Products: similarities and differences*

**Population dynamics of strain I-1237 of *Trichoderma atroviride* in Marlins soil**



Soil dilution technique followed by identification of strain I 1237 by PCR using the primers designed for the SCAR

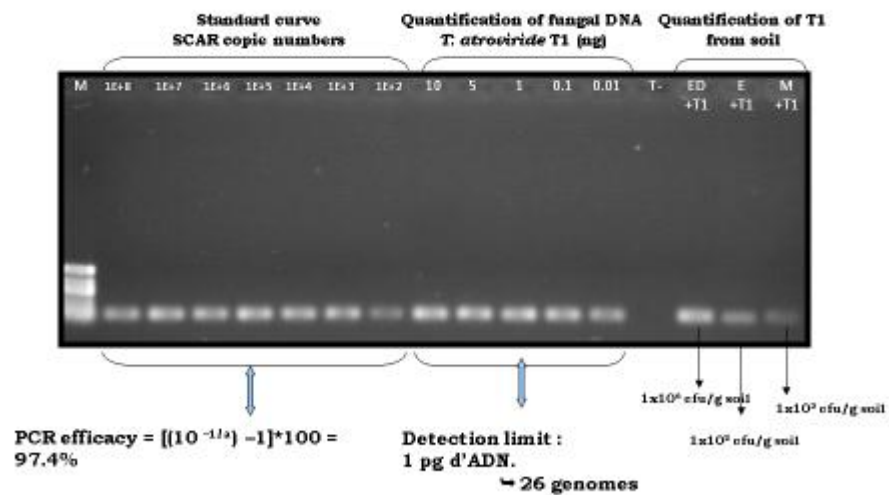
**In this acidic soil, the strain established at a level close to the lower inoculum concentration**

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- Another experiment was performed in which the population dynamics was followed using the qPCR technique.
- Total DNA was extracted from soil and the number of copies of SCAR marker was assessed by real time PCR

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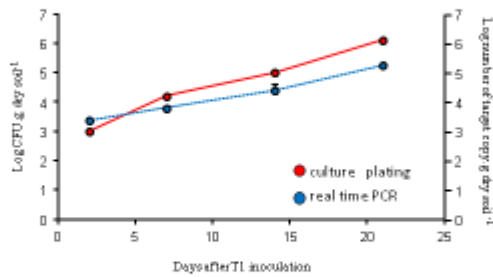
### Population dynamics of a strain of *Trichoderma atroviride* in soil by real time PCR using the SCAR marker



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**Sterilized soils inoculated with *T. atroviride* strain I-1237**

Epoisses soil



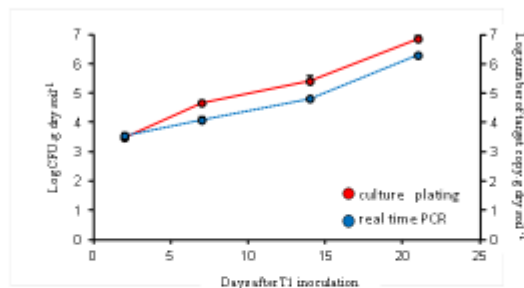
Similar dynamic pattern of I1237 population by real time PCR and culture plating

**After heat treatment, the population density increased, as expected in disinfected soil**

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**Sterilized soils inoculated with *T. atroviride* strain I-1237**

Marlins soil



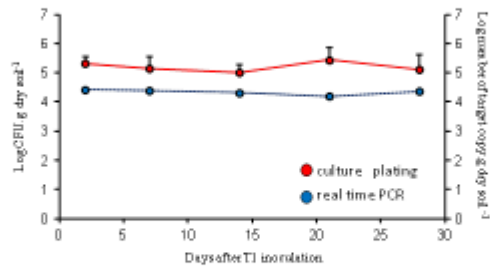
Similar dynamic pattern of I1237 population by real time PCR and culture plating

**After heat treatment, the population density increased, as expected in disinfected soil**

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### Natural soils inoculated with *T. atroviride* strain I-1237

Clay-loam soil from Epoisses pH=7



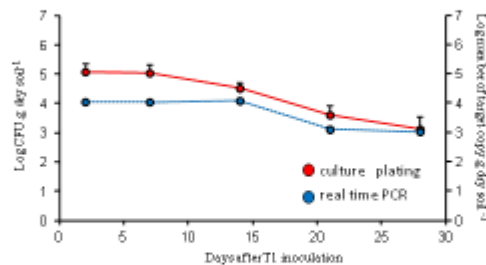
Similar dynamic pattern of T1 population by real time PCR and culture plating

**In this soil the introduced strain established at a population density close to the inoculation level**

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### Natural soils inoculated with *T. atroviride* strain I1237

acidic sandy soil from Marllins



Similar dynamic pattern of T1 population by real time PCR and culture plating

**In this soil the population density of the introduced strain first declined and then stabilized at a level two logs below the inoculation dose**

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- These data showed that strain I-1237 of *Trichoderma atroviride* actively colonized two sterilized soils, but in the same soils, in the presence of the autochthonous microbiota, it established at levels similar or lower than that of the inoculum concentration.
- The behaviour depends on the soil type, but the introduced strain does not become dominant.

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## **Effects of Trichoderma inoculation on the autochthonous microflora**

**To study the effects of introduction of I-1237 on the soil microflora we studied**

**1/ the effects on fungal and bacterial density**

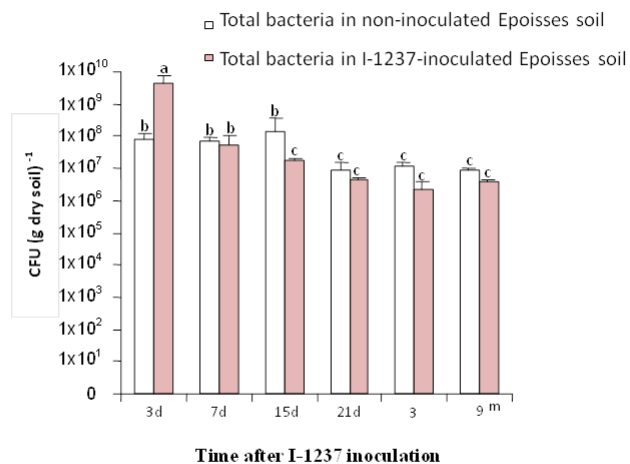
**2/ the effects on the structure of the fungal and bacterial communities**

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- In that experiment , the bacterial and fungal populations were assessed by the soil plate dilution technique and the density of I-1237 population among the total population of *Trichoderma* spp was determined using the SCAR marker.
- The structure of the microbial communities was assessed using the T-RFLP technique

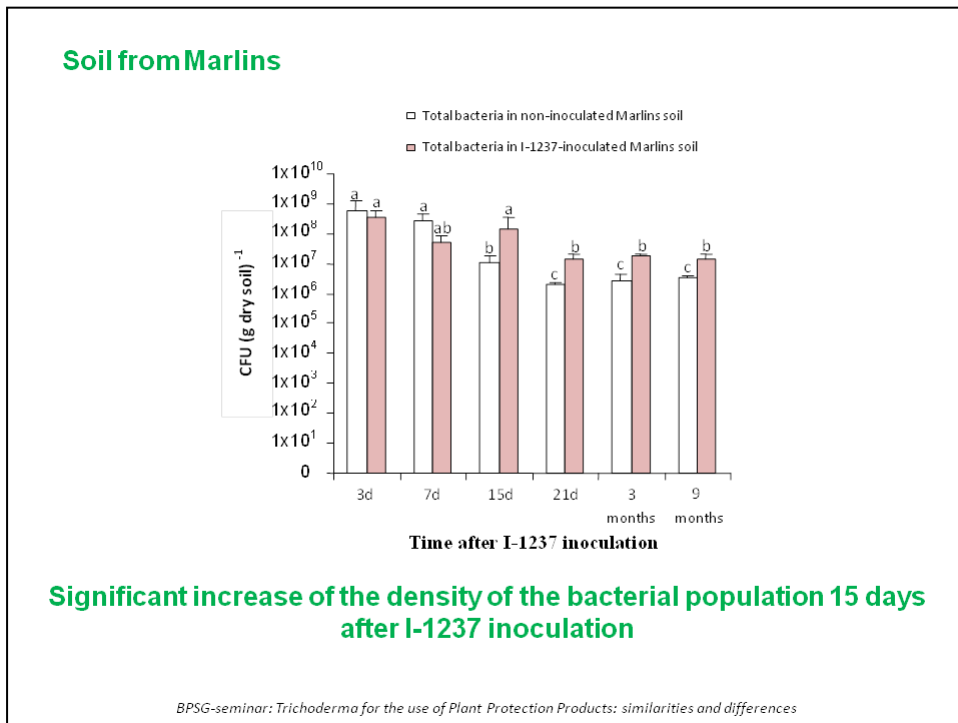
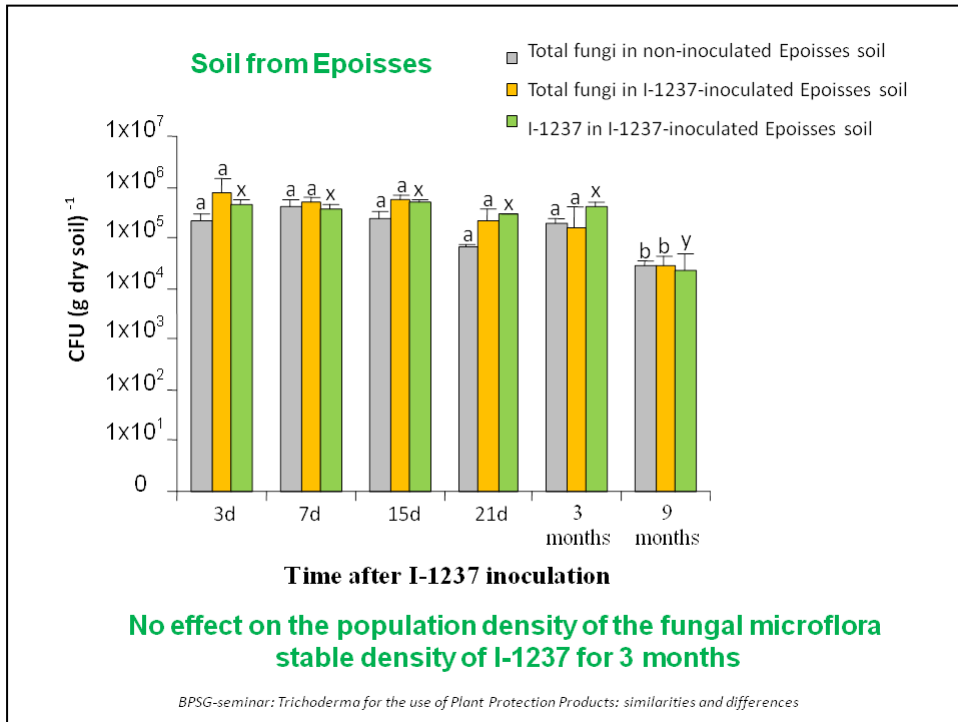
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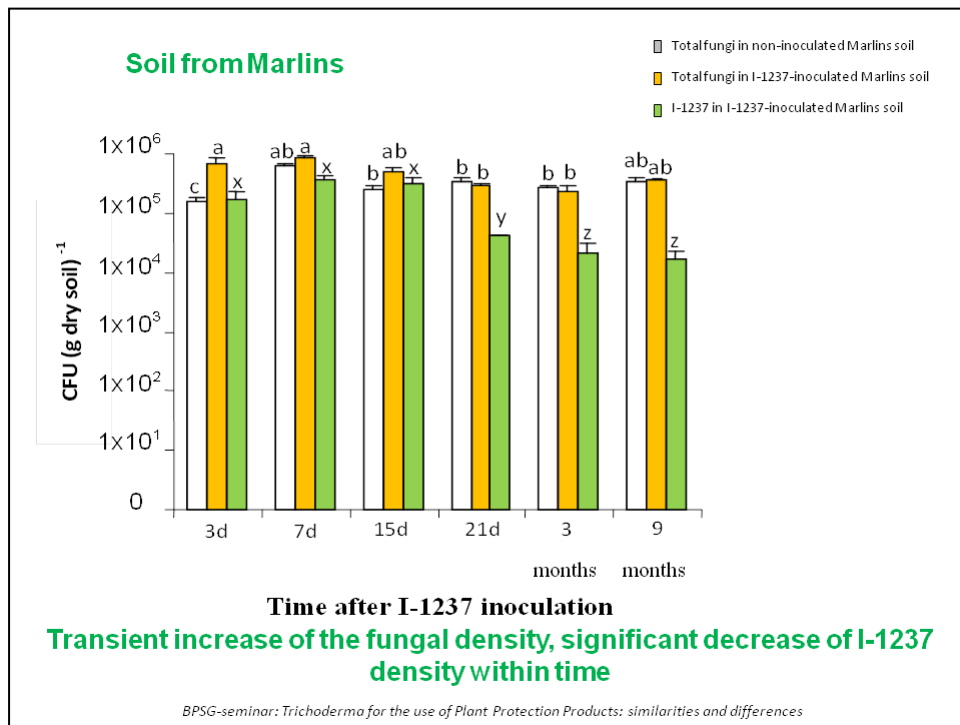
### Soil from Epoisses



**A transient effect was observed on the population density of bacteria**

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## Population density of I-1237 in soil over 9 months

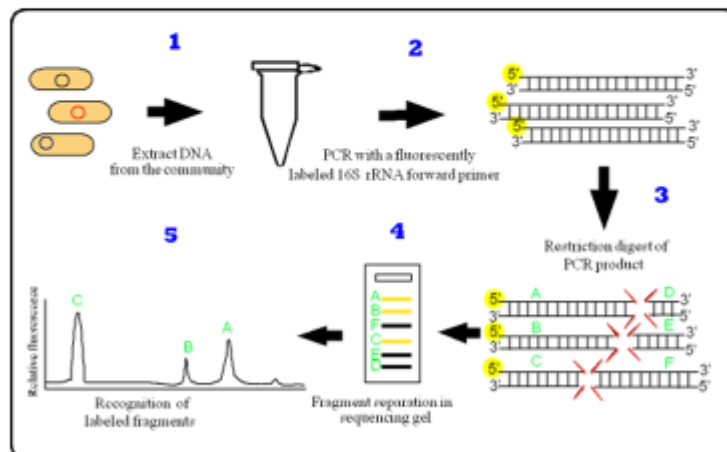
- I-1237 established in the soil from Epoisses at the density at which it was introduced,
- I-1237 established at a lower density in the acidic soil from Marlines

## Effect on the structure of the microbial communities

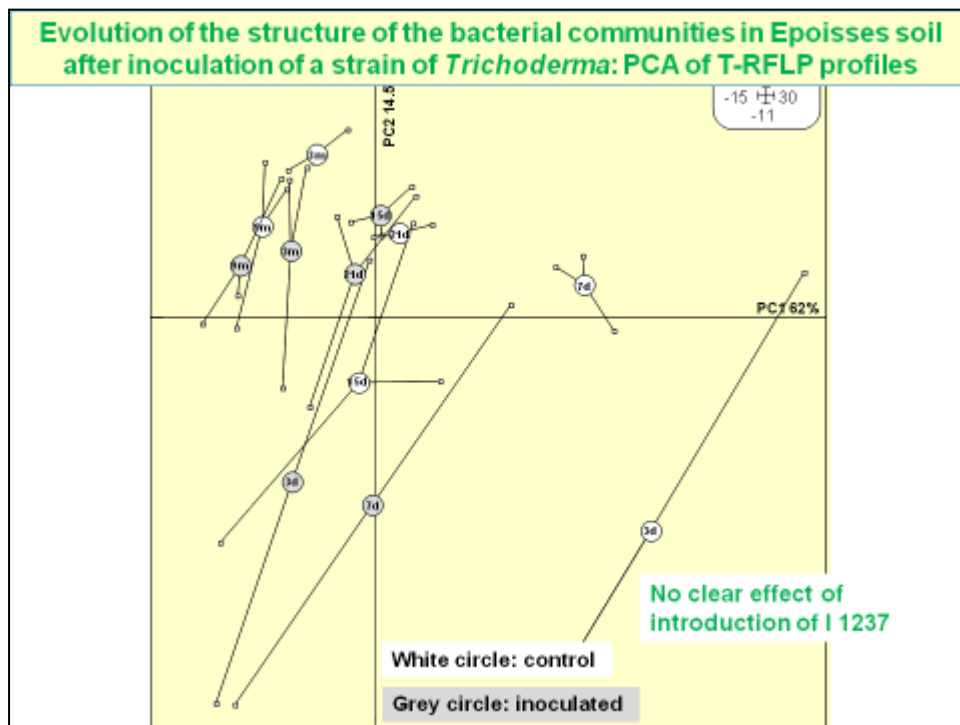
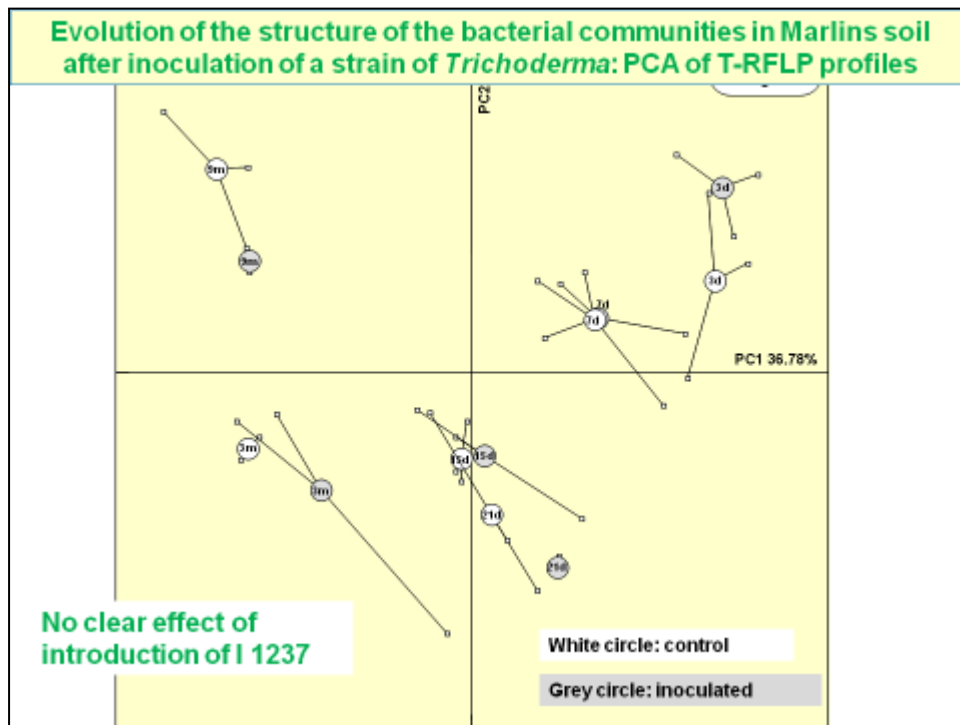
BPSG-seminar: Trichoderma for the use of Plant Protection Products: similarities and differences

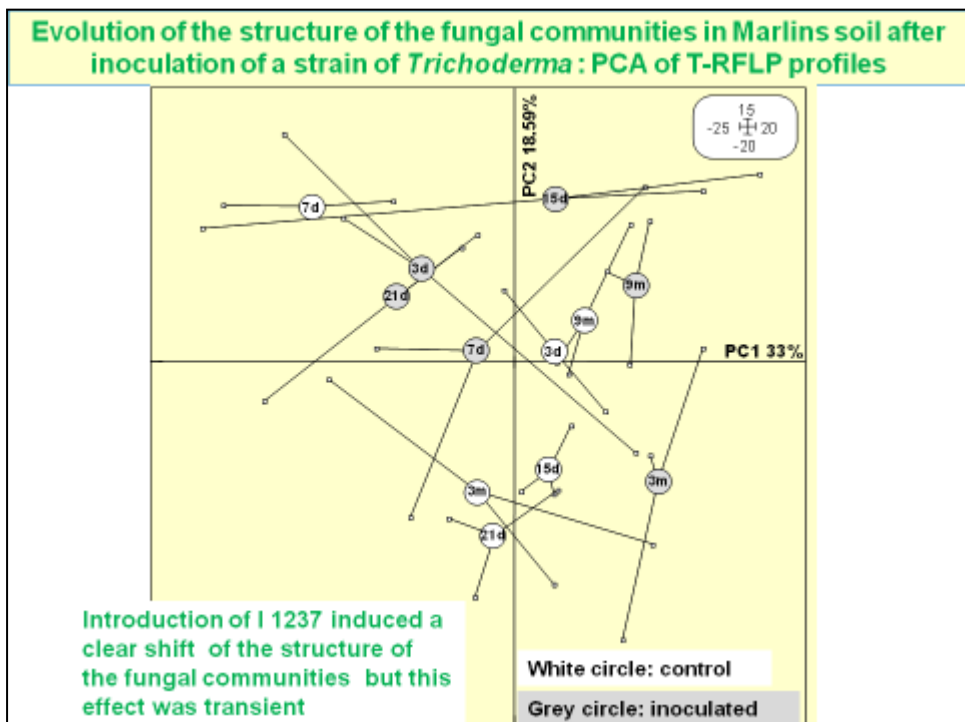
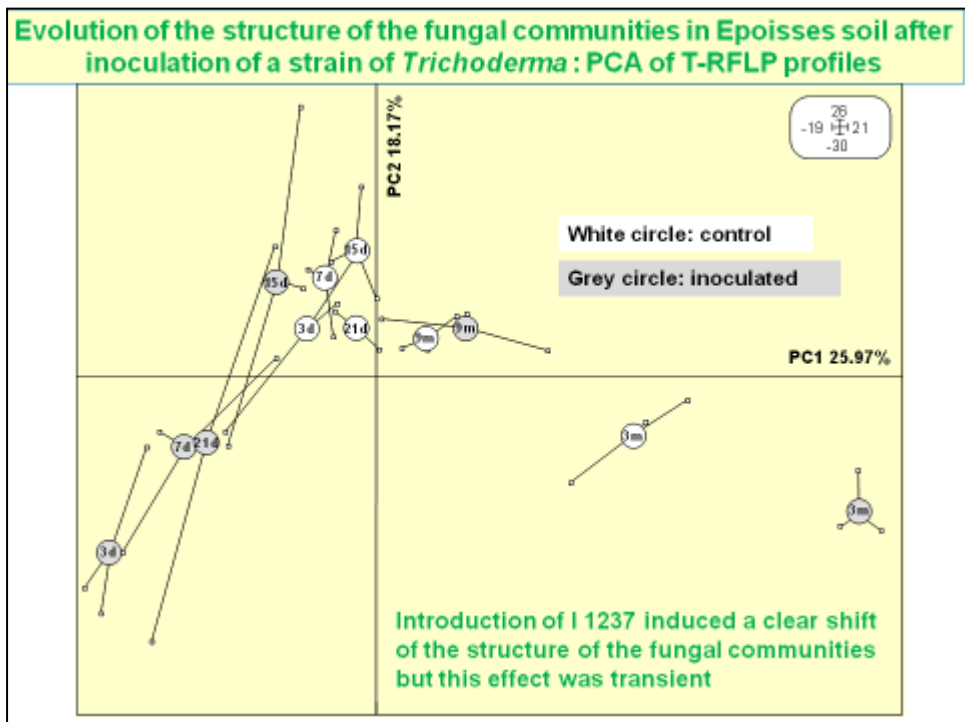
### STRUCTURE of the bacterial and fungal communities

#### • Terminal Restriction Fragment Length Polymorphism (T-RFLP)



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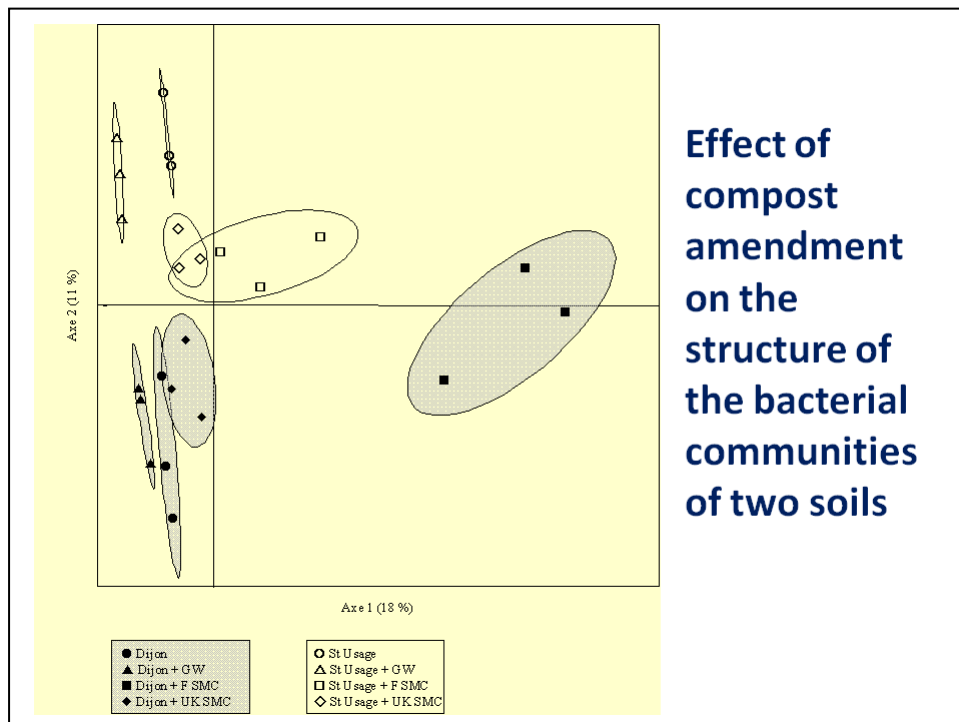
## **Effect of introduction of I-1237 on the structure of the microbial communities**

- No clear effect on the structure of the bacterial communities
- A clear but transient effect on the structure of the fungal communities
- The T-RF of strain I-1237 was detected among the fungal T-RF
- **The soils showed a good resilience to the introduction of this biocontrol agent.**

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## **Conclusion of a previous seminar**

- A soil microorganism re-introduced into soil will establish, but never become dominant in absence of any specific selection pressure
- As any other agricultural practice, MBCAs release has some effects on the soil microflora but these effects are always transients
- There should be no more concern for MBCA release than for manure or compost use



## Conclusion

- **The biocontrol strain I-1237 of *Trichoderma atroviride* will established in soils at different levels depending on the biotic and abiotic characteristics of the soils**
- **Its density will decline more or less rapidly, it never will become dominant**
- **Its effect on the autochthonous microflora is transient**
- **Its use does not present any specific risk in relation to the soil environment**

## References

- Cordier C, Edel-Herman V, Martin-Laurent F, Blal B, Steinberg C, Alabouvette C. 2007. SCAR based time PCR to identify a biocontrol strain of *Trichoderma atroviride* and study its population dynamics in soils. *Journal of Microbiological Methods* 68, 60-68
- Cordier C, Alabouvette C. 2009. Effects of the introduction of a biocontrol strain of *Trichoderma atroviride* on non target soil micro-organisms. *European Journal of Soil Biology* 45 267-274

**THANK YOU FOR YOUR ATTENTION**



BPSG-seminar: Trichoderma for the use of Plant Protection Products: similarities and differences

**Taxonomy, characterisation and identification of *Trichoderma*:  
experience in EU-evaluation and peer review**

**[PPT 7]**

*Kersti Gustafsson (Swedish Chemicals Agency - Kemi; Sweden)*

Taxonomy, characterisation and identification  
of *Trichoderma*: experience in EU-evaluation  
and peer review

*Kersti Gustafsson*

Swedish Chemicals Agency - KEMI Sweden

2012-06-11

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Regulatory background  
and latest developments

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## Regulatory background

- Commission Regulation (EC) No 2229/2004 of 3 December 2004 laying down further detailed rules for the implementation of the fourth stage (List 4) of the programme of work referred to in Article 8(2) of Council Directive 91/414/EEC
- Commission Directive 2008/113/EC of 8 December 2008 amending Council Directive 91/414/EEC to include several micro-organisms as active substances (in Annex 1)
- The peer review process is now ongoing

EC = European Commission

EEC = European Economic Community

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## SE RMS for *Trichoderma harzianum* on "List 4"

### Task Force Dossier for

*Trichoderma harzianum* strains T-11, T-22, ICC012, ITEM 908

### Annex 1 inclusion

- ***Trichoderma asperellum*** (formerly *T. harzianum*) strain ICC012
- ***Trichoderma atroviride*** (formerly *T. harzianum*) strain T11
- ***Trichoderma harzianum*** Rifai strain T-22
- ***Trichoderma harzianum*** Rifai strain ITEM 908

RMS = Rapporteur Member State

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## Latest developments — The ongoing peer review

- Written commenting round 2011
- Response to comments 2011/2012 from both the notifier and RMS
- RMS addenda to the DAR
- EFSA opinion is awaiting

RMS = Rapporteur Member State  
DAR = Draft Assessment Report  
EFSA = European Food Safety Authority

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## Morphology and taxonomy (genotyping) of *Trichoderma spp*

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## Taxonomy in brief - *T. harzianum* Rifai

- Rifai (1969) proposed nine species aggregates (*T. piluliferum*, *T. polysporum*, *T. hamatum*, *T. koningii*, *T. aureoviride*, *T. harzianum*, *T. longibrachiatum*, *T. pseudokoningii* and *T. viride*)
- The taxonomic classification still valid when the Council Directive 91/414/EEC became effective in July 1993.

## *T. harzianum* Rifai genotyping

- Comparison of ITS1 sequences (2000) from different biocontrol isolates of *Trichoderma* spp. phylogenetic tree with five groups
  - *T. harzianum* / *T. inhamatum*; biocontrol strains
  - Mushroom competitors;
  - *T. paracerosum* / *T. longibrachiatum*;
  - *T. hamatum* / *T. asperellum*; biocontrol strains
  - *T. viride*, *T. atroviride*, and *T. koningii*. biocontrol strains
- Phylogenetic analyses (2004) with 53 *Trichoderma* isolates using the ITS1 region and a part of the *tef* gene.
  - The analyses lead to a finer resolution of the phylogeny within the genus *Trichoderma*. Phylogenetic trees for both genes showed biocontrol isolates in all groups except *T. aggressivum*.

ITS = internal transcribed spacer; *tef* = translation elongation factor

## RMS SE comments

- Taxonomy in general in a revolutionizing period with molecular biology and DNA techniques
- *Trichoderma harzianum* on List 4 - all four strains in the compiled dossier originally belonged to *Trichoderma harzianum* Rifai
- Development of oligonucleotide barcodes for species identification (2005) - possible to strictly identify the four strains to species level
- Checking the gene sequences with a gene bank, NCBI (National Centre for Biotech Information), the following species identification was the result:

**T-22** and **ITEM 908** are *Trichoderma harzianum* = *Hypocrea lixii*

**ICC012** is *Trichoderma asperellum sensu* Hermosa *et al* 2000

**T-11** is *Trichoderma atroviride sensu* Hermosa *et al* 2000

## T-22

- **T-22** protoplast fusion (1988) from mutants of T-12 and T-95
- **T-22** have a combination of biocontrol capacities of T12 originally isolated from USA and of rhizosphere colonisation capacities of T-95 isolated from Colombia.
- Protoplast fusion ~ anastomosis
- EU Commission response
  - The organism is formed by protoplast fusion of organisms, which are capable of exchanging genetic material by traditional breeding methods; it should therefore on this basis be exempt from Directive 2001/18/EC

EU = European Union

## Genotype *versus* phenotype

-

### T-11, T-22, ICC012, ITEM 908

- Taxonomically different genotypes – different species
- Strain specific information compared even though there are no specific differences found yet - these four strains treated as one species, read across between information/tests
- Discussed at an EFSA expert meeting, temperature range is not possible to read across

## Read across

-

### Listing of endpoints

- Read across between strains acceptable
- Listing of endpoints separate, as strain specific inclusion in Annex 1

## Mode of action and secondary metabolites

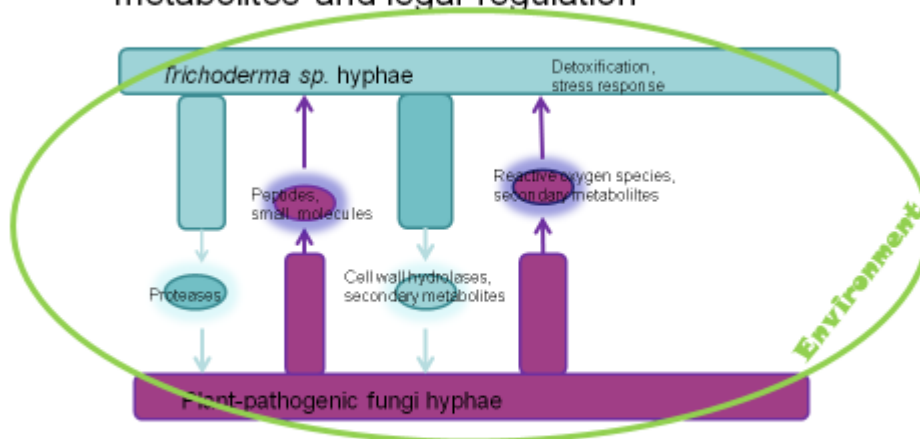
## Information in the DAR

- *Trichoderma spp.* are able to produce a lot of different metabolites belonging to polyketides, sesquiterpenes (including the mycotoxin group of trichothecenes), viridofungins, and peptaibols.
- Accumulation of potentially toxic metabolites in the environment was not observed to date and effects of antibiosis against bacteria or fungi were only demonstrated *in vitro*.
- Toxicity of metabolites to mammalian cells was only demonstrated with purified metabolites.

## Data gaps proposed by EFSA

- Identify all mammalian toxins/secondary metabolites produced by the respective strain both in the product and when applied to the crop
- Specific data on the toxins produced with quantification in the crops and a consumer risk assessment

## Mode of action of importance related to metabolites and legal regulation



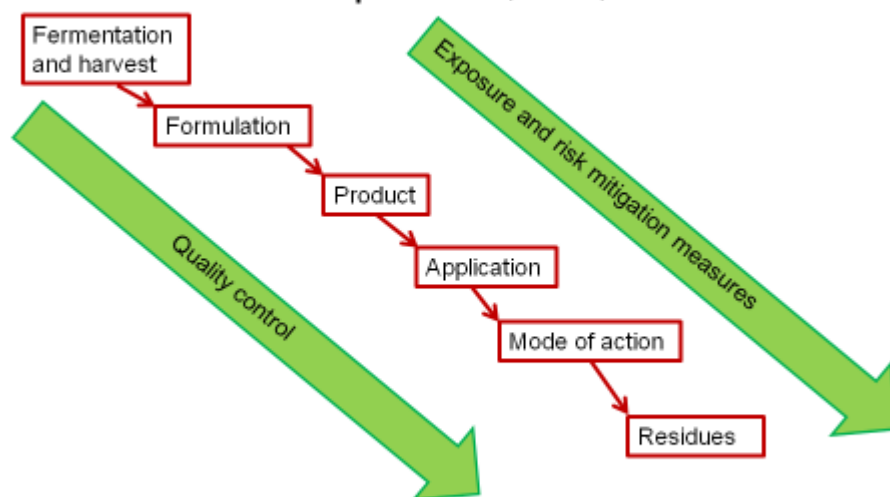
Simplified from Druzhinina et al. 2011. Nature Reviews Microbiology 9:749-759

## Pragmatic reasoning on metabolites

A pragmatic view discussed at an EFSA expert meeting and recommendations were agreed by the experts:

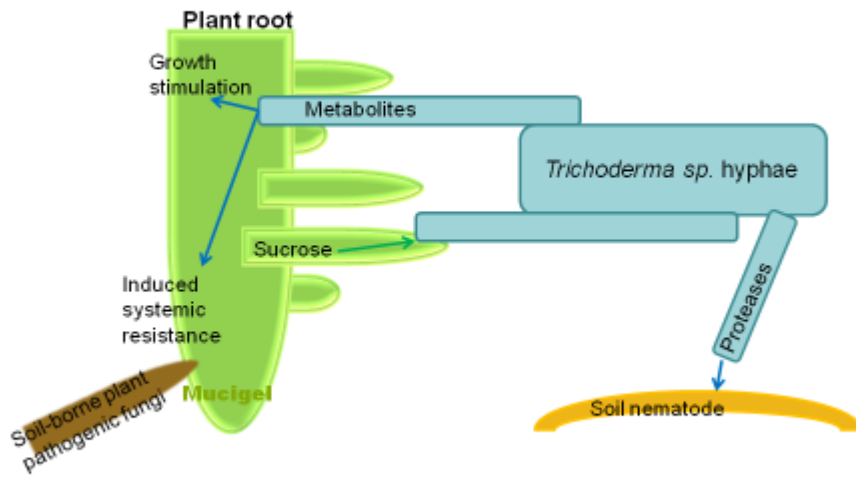
- Metabolites/toxins only **produced** during fermentation and/or at growth. Human and environmental **exposure** expected to be low - unless there are residues from the fermentation process in the product.
- A pragmatic approach
  - no effects seen in the available toxicity tests
  - no indication is available from public literature
  - no relevant metabolites expected
- Data requirement: metabolites need to be assessed
  - if related to mode of action
  - present in significant amounts under practical conditions of use and not related to the mode of action
- Data requirement: Quality assurance criteria for production should be submitted

## Production process, use, residues



## Side effects on the soil microflora

### Some interactions with non-target organisms in the rhizosphere



Simplified from Druzhinina *et al.* 2011. *Nature Reviews Microbiology* 9:749-759

## No effect levels

Species and end point	NOEC
<i>Daphnia magna</i> cumulative values of total number of alive, young reproduced by all adults	$6.0 \times 10^7$ CFU/L
<i>Desmodesmus subspicatus</i> growth rate and biomass	$6.5 \times 10^8$ CFU/L
<i>Typhlodromus pyri</i> reproduction rate	$< 3.7 \times 10^9$ CFU/ha

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Mean application rate	$10^{12}$ CFU/ha
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CFU = colony forming units

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## Further aspects on *Trichoderma* spp.

- ♠ further data gaps/comments
- ♠ the continuing EU process
- ♠ equivalence check

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## Further data gaps / comments

- The quantitative method for the technical and formulated products must be validated.
- Batch data should be provided to show compliance with the OECD Issue Paper dated February 2011 'Discussion on Microbial Contaminant Limits for Microbial Pest Control Products'.

## The continuing EU process

### EFSA conclusion

- List of studies to be generated, still ongoing or available but not peer reviewed
  - A complete list of data gaps identified during the peer review process, including those areas where a study may have been made available during the peer review process but not considered for procedural reasons (in case of **potentially harmful effects** the study can be evaluated).

## Confirmatory data, revision of review report and Annex 1 inclusion decision

COM proposes confirmatory data ≠ List of studies to be generated, still ongoing or available but not peer reviewed

- Decision with time schedule
  - Deliver within a certain time frame
  - Will be handled at product authorisation
  - Will be handled at re-evaluation
  
- Revision of review report and Annex 1 inclusion decision

COM = Commission

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## Equivalence check of Trichoderma

SANCO/10597/2003 rev. 8.1, *Guidance document on the assessment of equivalence of technical materials of substances regulated under Council Directive 91/414/EEC*

Guidance document for chemical active substances

- assessing the equivalence of different sources of technical material versus the reference source

Basis for the assessment of the microorganisms

SANCO = European Commission Directorate General Health and Consumers

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## *Trichoderma harzianum* T-22

- Five batches analyses
  - Comparative identification: comparison of the sequence the ITS region and also the *tef* gene
  - Content of active micro-organism *T. harzianum* T-22 in batch analyses
  - Control of contaminants
- Method descriptions

MS decision on equivalence

MS = Member State


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**An overview of regulatory data versus literature data in mammalian toxicology, environmental fate and ecotoxicology in view of a discussion about bridging versus strain/species specific data  
[PPT 8]**


*Christine Vergnet (French Agency for Food, Environmental and Occupational Health & Safety [ANSES], Paris; France*




**Trichoderma:**  
**Regulatory data versus literature data**  
Mammalian toxicology  
Environmental fate  
Ecotoxicity


K. ANGELI and C. VERGNET

Seminar on « *Trichoderma* spp. for the use in plant protection products: similarities and differences »,  
OECD, PARIS, Monday 11 June 2012



- *Trichoderma* new (2 strains):
  - Peer reviewed
  - Efsa conclusions available
- *Trichoderma* List 4 (9 strains):
  - Annex I (green track)
  - under peer review



Species (submission)	New name	Strain	Notifier Products	Products
Trichoderma harzanium (list 4)	Trichoderma atroviride	IMI 206040	BINAB Bio-Innovation AB BINAB TF WP BINAB T vector	BINAB TF WP BINAB T vector
Trichoderma polysporum (list 4)		IMI 206039		
Trichoderma harzanium RIFA (list 4)	Trichoderma atroviride	T-11	Newbiotechnic	TUSAL T. harzanium T11 + Tviride T25
	Trichoderma harzanium	T-22	Koppert BioWorks	TRIANUM-P et -G F-STOP
	Trichoderma harzanium	ITEM908	Agnifutur	TRICOVER-G et WP
	Trichoderma asperellum	ICC012	Isagro	REMEDIER (notin vol 1)
Trichoderma viride (list 4)	Trichoderma gamsii	ICC080	Isagro	
	Trichoderma asperellum	T-25	Newbiotechnic	TUSAL T. harzanium T11 + Tviride T25
	Trichoderma asperellum	TV1	Agribiotech	VIRISAN
Trichoderma atroviride (new)		I-1237	Agrauxine	ESQUIVE WP
Trichoderma asperellum (new)		T34	Biocontrol Technologies S.L.	T34 Biocontrol Usage 

### Literature data (*Trichoderma sp.*)

- **Toxicology:**

- medical data and direct observation:
  - pathogenic isolates restricted mainly to *T. longibrachiatum*
  - few cases in immunosuppressed patients but no relevant route of administration and immunocom
  - no case from *Trichoderma* biocontrol agents
- medical surveillance on manufacturing plant personnel:
  - possible effects from exposure in greenhouse

- **Fate and behaviour in the environment:**

- Frequently the most prevalent soil fungus, variable distribution of the species, associated with the rhizosphere, abiotic and biotic factors for survival and development, introduced conidiospores remains dormant and decline in bulk soils
- Present in surface water but not an aquatic fungus, limited survival of introduced species
- Present in air but is not a dominant fungus
- Low mobility of conidiospores through soil (other fungi)

### Literature data (*Trichoderma* sp.)

- **Ecotoxicology (ecology):**
  - No case of infectiosity in fish
  - Microorganisms: transient effects on microbial communities
  - *T. harzanium* / *Bombus terrestris*: no infectivity, no pathogenicity, no effect on brood development, no effect on bumble bees used as dispersal agent
  - *T. harzanium* / *Apis mellifera*: no effect on brood development, no effect on bees used as dispersal agent for 81 to 97 days
  - *T. harzanium* / Non target arthropods (*Orius insidiosus*, *Macrolophus caliginosus*, *Phytoseiulus persimilis*, *Encarsia formosa*): no infectivity, no pathogenicity
  - *T. viride* used as a source of food for *Orius laevigatus* and *Neoseiulus cucumeris*
  - A strain of *T. viride* described to be pathogenic to germinating seedling but after research low level infection under one condition
- **Mode of action of *Trichoderma* biocontrol agents:**
  - Colonisation of the rhizosphere when active
  - Mycoparasitism when active
  - Secretion of antifungal substances when active
  - Plant growth promotion when active
  - Induction of systemic resistance when active



### *T. atroviride* I-1237: data in toxicology

- Toxicity/pathogenicity/infectivity studies:
  - Oral adm.: no effect, complete clearance
  - Intra-tracheal adm.: no effect, complete clearance
- Toxicity studies:
  - Subcutaneous adm.: no effect
  - Eye and skin irritation (product): no effect
- Genotoxicity studies (Ames, micronucleus in vivo): negative
- No growth > 35° C (optimum: 20-25° C)
- Medical surveillance on manufacturing plant personnel: no sensitizing and /or allergenic incident
- No relevant toxin (10 trichothecenes not found in MPCA nor MPCP)
  
- Data gaps identified by Efsa:
  - information regarding the production of toxins or secondary metabolites



### ***T. asperillum* T34: data in toxicology**

- Toxicity/pathogenicity/infectivity studies:
  - Oral adm.: some effects, complete clearance
  - Intra-tracheal adm.: some effects, **deaths** (high volume and route adm.), complete clearance
  - Intra-peritoneal adm.: some effects, **deaths** (high volume and route adm.), complete clearance, death not confirmed in 2 add. studies, no effect with sterile filtrate medium
- Toxicity studies (product):
  - Acute oral, cutaneous, inhalation: no effect
  - Eye and skin irritation: no effect
- Genotoxicity studies (Ames) : no effect (supernatant of product)
- No growth > 36° C
- No relevant toxin in the product (T2, Harzianum A not found and trichodermin unlikely at relevant level), antibiotic or antifungal unlikely
- medical surveillance on manufacturing plant personnel: no effect (T34)
  
- Data gap identified by Efsa:
  - information regarding the production of toxins or secondary metabolites



### ***T. viride*: data in toxicology**

#### ***T. gamssi* ICC080**

- Toxicity/pathogenicity/infectivity studies :
  - Intra-tracheal adm.: no toxicity, no pathogenicity, complete clearance
- Toxicity studies :
  - Oral adm.: no toxicity
  - Intra-peritoneal adm.: no toxicity
- No growth > 35° C
- Medical surveillance on manufacturing plant personnel: no effect

#### ***T. asperillum* T25**

- No growth > 35° C , optimum: 20 – 28° C
- Medical surveillance on manufacturing plant personnel: no effect

#### ***T. asperillum* TV1**

- Toxicity studies (product):
  - Acute oral, cutaneous: no effect
  - Eye and skin irritation: no effect
  - Acute inhalation (post DAR) : no effect
- No growth > 35° C , optimum: 20 – 28° C (post-DAR)
- Medical surveillance on manufacturing plant personnel: no effect



### ***T. harzanium* RIFAI: data in toxicology**

#### ***T. asperellum* ICC012**

- Toxicity/pathogenicity/infectivity studies :
  - Intra-tracheal adm.: no toxicity, no pathogenicity, complete clearance
- Toxicity studies :
  - Oral adm.: no toxicity
  - Intra-peritoneal adm.: no toxicity
- No growth > 35° C , optimum: 20 – 30° C
- Medical surveillance on manufacturing plant personnel: no effect

#### ***T. atroviride* T-11**

- Toxicity studies :
  - Oral adm.: no toxicity
  - Intra-tracheal adm.: no toxicity
  - Intra-peritoneal adm.: no toxicity
- No growth > 35° C , optimum: 20 – 28° C
- Medical surveillance on manufacturing plant personnel: no effect

### ***T. harzanium* RIFAI: data in toxicology**

#### ***T. harzanium* T-22**

- Toxicity /pathogenicity /infectivity studies :
  - Oral adm.: no toxicity, no pathogenicity, complete clearance
  - Intratreacheal adm.: no toxicity, no pathogenicity, complete clearance
  - Intravenous adm.: no toxicity, mild pathogenicity, complete clearance
- Toxicity studies:
  - Subcutaneous adm.: no toxicity
  - Eye irritation: slight effect
- No growth > 35° C , optimum: 20 – 28° C (post-DAR)
- Genotoxicity studies (Ames with filtered supernatant , micronucleus in vivo) : no effect
- Medical surveillance on manufacturing plant personnel: no effect

#### ***T. harzanium* ITEM908**

- Medical surveillance on manufacturing plant personnel: no effect

### ***T. harzanium (ex viride)* BINAB: data in toxicology**

#### ***T. atroviride* IMI 206040 & *T. polysporum* IMI 206039 in the product**

- Toxicity studies (product):
  - Oral adm.: no toxicity (indicative, old study, unclear test item)
- IMI 206040: No growth > 34° C , optimum: 20 – 30° C
  - IMI 206039: No growth > 30° C , optimum: 20 – 25° C
  - No relevant toxin in product (trichothecenes)
- Medical surveillance on manufacturing plant personnel: no effect

PRAPeR M5 proposes to require:

- a toxicity/infectiosity/pathogenicity study (intratracheal adm.)
- an acute oral toxicity study with the product BINAB TF WP



### ***T. atroviride* I-1237 / ESQUIVE WP: e-fate data**

- Ability to colonize soils, rhizosphere and plant roots
  - Colonisation of carrots in the condition of use
  - Influence of soil parameter on the strain development
    - Temperature, pH, Organic matter
  - Viability in water (96-h fish test)
- 
- Data gaps identified by Efsa:
    - information regarding the production of toxins or secondary metabolites



### ***T. asperillum* T34 / T34 Biocontrol: e-fate data**

- Dried conidies to be mixed with compost or dipping water
- Presence in substrates under condition of use (efficacy study): development and maintenance
- Mobility under condition of uses (efficacy study): decreasing density with increasing depth of soil, present in the drained water after pots being flooded
- Fate in microcosm simulating surface and ground water (filter-sterilized): survival, very few germination
- Air after 2 year drip irrigation in a greenhouse: very low prevalence
- Data gaps identified by Efsa:
  - information regarding the production of toxins or secondary metabolites
  - Fate and behaviour in soil and water (disposal of growth media, non permanent structure)

anses 

### ***T. viride* : e-fate data**

#### ***T. gamssi* ICC080**

- No strain specific data

#### **REMEDIER (*T. gamssi* ICC080 + *T. asperillum* ICC012)**

- Rhizosphere competence and population distribution in soil environmental compartments in eggplants

#### ***T. asperillum* T25**

- No strain specific data

#### ***T. asperillum* TV1**

- No strain specific data

anses 

### ***T. harzanium* RIFAI: e-fate data**

***T. asperellum* ICC012**

- No strain specific data

**REMEDIER (*T. gamssi* ICC080 + *T. asperellum* ICC012)**

- Rhizosphere competence and population distribution in soil environmental compartments in eggplants

***T. atroviride* T-11**

- No strain specific data

***T. harzanium* T-22**

- No strain specific data

***T. harzanium* ITEM908**

- No strain specific data

### ***T. harzanium (ex viride)* BINAB: e-fate data**

***T. atroviride* IMI 206040**

- No strain specific data

***T. polysporum* IMI 206039**

- No strain specific data

**Air compartment:**

- CFU concentrations in housing and manufacturing facilities (indicative)

### ***T. atroviride* I-1237 / ESQUIRE WP: data in ecotoxicology**

- Fish-96 h, daphnid-48 h, alga-72 h : no effect
- Bee-48 h : no effect
- NTA (Aphidius, Typhlodromus) : no effect
- Earthworm-14 d : no effect; earthworm-56 d post-DAR: no effect
- Transient effects on microbial communities
- Small effect on symbiosis
- Plant: no phytotoxicity, no pathogenicity (efficacy trials on carrot, grape, salad)
  
- Data gaps identified by Efsa:
  - information regarding the production of toxins or secondary metabolites
  - Pathogenicity and infectivity (aquatic org., bee, NTA)
  - N and C mineralisation

### ***T. asperullum* T34 / T34 Biocontrol : data in ecotoxicology**

- no effect on microbial community structure (indoor use)
  
- Data gaps identified by Efsa:
  - information regarding the production of toxins or secondary metabolites
  - Ecotox risk assessment (disposal of growth media, non permanent structure)
  - Effect on sewage organisms

### ***T. viride* : data in ecotoxicology**

#### ***T. gamssi* ICC080**

- Fish-30 d, daphnid-21 d, alga-72 h, Lemna-7 d : no effect
- Bee-48 h : no effect
- Earthworm-14 d : no effect
- C & N mineralisation: no effect

#### **REMEDIER (*T. gamssi* ICC080 + *T. asperellum* ICC012)**

- *Typhlodromus pyri*: no effect
- No pathogenicity to crops

#### ***T. asperellum* T25**

- No strain specific data

#### ***T. asperellum* TV1**

- No strain specific data

### ***T. harzanium* RIFAI : data in ecotoxicology**

#### ***T. asperellum* ICC012**

- Fish-30 d, daphnid-21 d, alga-72 h, Lemna-7 d : no effect
- Bee-48 h : no effect
- Earthworm-14 d : no effect
- C & N mineralisation: no effect

#### **REMEDIER (*T. gamssi* ICC080 + *T. asperellum* ICC012)**

- *Typhlodromus pyri*: no effect
- No pathogenicity to crops

#### ***T. atroviride* T-11**

- No strain specific data

#### ***T. harzanium* T-22**

- No strain specific data

#### ***T. harzanium* ITEM908**

- No strain specific data

### ***T. harzanium (ex viride)* BINAB: data in ecotoxicology**

#### ***T. atroviride* IMI 206040 & *T. polysporum* IMI 206039 in MPCP**

- No effect in the BINAB Eco-Monitor (aquatic and terrestrial cosm, 14 years)
- No effect on *Bombus terrestris* used as dissemination vectors

- PRAPeR M5 proposes to require:
  - a 21 d –test on *Daphnia magna*

### **Literature versus regulatory data for *Trichoderma* sp**

- A large strain specific data set was provided for new strains of *Trichoderma* sp. (*T. atroviride* I-1237 and *T. asperellum* T-34)
- Depending on the sections, strain specific data were not equally provided for the 4 strains grouped as *T. harzanium* RIFAI and the 3 strains grouped as *T. viride*
- The four strains of *T. harzanium* RIFAI are now separated into 3 species (*T. harzanium*, *T. atroviride*, *T. asperellum*)
- The 3 strains of *T. viride* are now separated into 2 species (*T. gamssii*, *T. asperellum*)
- The two strains of the BINAB product (*T. atroviride ex harzanium* and *T. polysporum*) are said to be initially classified as *T. viride*, few product specific studies were provided but of interest to assess effects of non-target species

### Literature versus regulatory data for *Trichoderma* sp

- Bridging between strains was supported by the applicants (for the strains initially submitted together)
- Literature data is generally not strain specific , relates to species according to the previous classification and sometimes is not species specific

## Proposed focus for discussion

**Criteria to bridge between strains / species to be set**

**Which strain specific data (toxicology  
environmental fate and ecotoxicity ) are necessary ?**

**Task Force I: Experiences with the registration of *Trichoderma harzianum*: active substance and products, in various European countries**

[PPT 9]

*Willem Ravensberg (Koppert Biological Systems, Berkel & Rodenrijs; The Netherlands)*



**Task Force T. harzianum:  
Experiences with the registration of *Trichoderma harzianum*: active substance and products, in various European countries**



**Willem Ravensberg**  
Koppert  
Chairman Task Force T. harzianum




**Why was a Task Force formed?**

- EC Regulation 2229/2004
- Fourth stage review programme, Part C: microorganisms
- Dossier submission 30 November 2005
- **EU recommends to form a task force**
- Legislative position? Obligatory?
- Less dossiers to evaluate
- Less costs
- Industry cooperation required, Consortium Agreement
- Initially 8 companies, finally 4 companies in TF

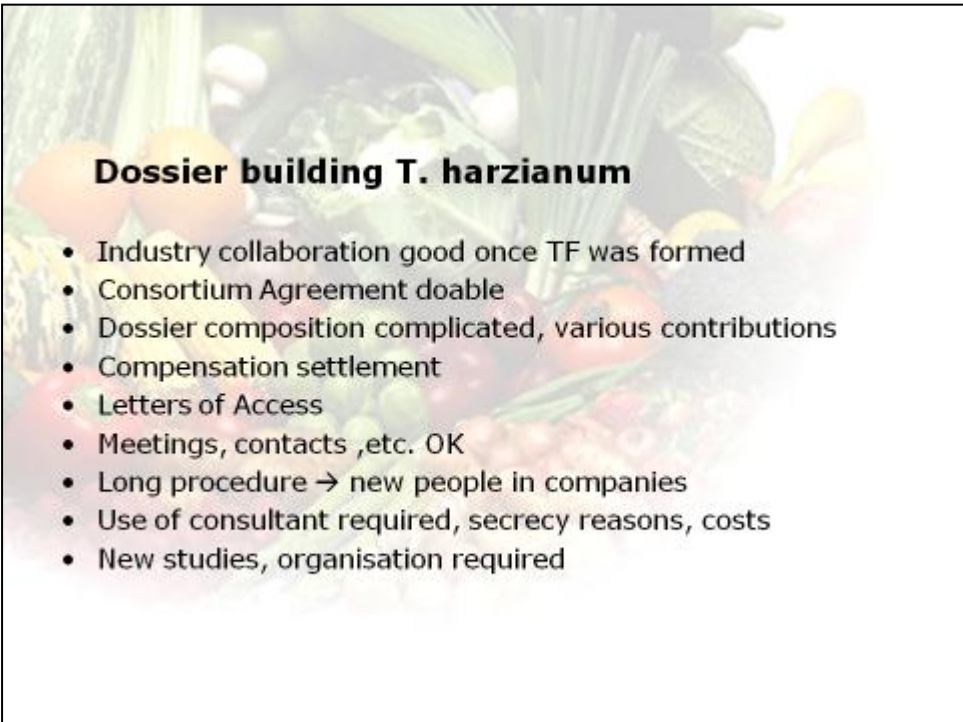
  

- Annex I inclusion is on strain level
- Task force: combination of strains of one species
- From onset approval of EU on this approach



### **RMS Sweden designated for *T. harzianum***

- One-sided decision, no industry involvement
- No product registration of TF members in RMS
- High fees/costs
- Experienced authority? One product approved
- Lead rapporteurs for microorganisms: Sw and NI → role??
- Procedural: two pre-submission meetings, good contacts by phone, emails, etc.
- Invoices had to be prepaid



### **Dossier building *T. harzianum***

- Industry collaboration good once TF was formed
- Consortium Agreement doable
- Dossier composition complicated, various contributions
- Compensation settlement
- Letters of Access
- Meetings, contacts ,etc. OK
- Long procedure → new people in companies
- Use of consultant required, secrecy reasons, costs
- New studies, organisation required

## Task Force Position Paper

### Position Paper sent to DG SANCO (nov 2005)

- Re-registration : asked for exemption of recent approved products
- Comments on choice and designation of RMS
- Various levels of registration fees between MS's
- Transparency and unpredictability of costs and fees
- Trichoderma and its various positions in MS's
- Consequences of these issues for industry
- Solutions suggested for these issues
  
- → no response from DG SANCO

## RMS, DAR and review process

- |                                |                    |
|--------------------------------|--------------------|
| • 2 Pre-submission meetings    | 2005               |
| • Dossier submission           | Nov 2005           |
| • Completeness check           | Feb 2006           |
| • Writing of DAR               | 2006- July 2007    |
| • Questions to THTF            | Dec '06, April '07 |
| • DAR publication by EFSA      | May 2008           |
| • Annex Inclusion              | 2009               |
| • MS, EFSA, TF comments on DAR | 2010-2011          |
| • Eval. tables commented by TF | 2011               |
| • EFSA review ongoing          | Dec 2012 ??        |

### Issues with active substance

- **Strain Th T22 a GMO or not?** RMS and DG Environment agreed it is not a GMO, protoplast fusion (like in plants) is a natural process. Exempt of 2001/18/EC – “Deliberate release of genetically modified organisms”
- Still questions of MS’s and EFSA. Unofficial procedure ?? Needs to be clarified and decided. Koppert has not had any official report on the conclusion.
- Re-classification of 2 out of 4 strains: *T. asperellum*, *T. atroviride*: relatedness of strains, read across between strains; what is needed to ascertain comparability?
- Data required for all products in stead of one representative formulation for all strains, GAP information for all products
- Contaminants
- Metabolites
- Genotoxicity of microorganisms/metabolites: testing methods?
- Physical - chemical properties

### Issues at national level

- Provisional approval of a.s. for Annex I inclusion (“green light” category)
- Categorized as a “biofungicide” while TF asked for plantstrengthenener/biofungicide, no feedback received
- National authorizations to be done without a final risk assessment (EFSA) and decision (EU)
- Various interpretations of EU and national legislation
- Consequences: extra costs, extra long procedures, different outcomes
- Product re-registration: deadline April 30, 2012, notifiers had to submit dossiers without having access to the EFSA review and final conclusion

## Products at national level

### **Trianium - Koppert: one dossier, one label:**

*T. harzianum* T22

- |               |   |             |
|---------------|---|-------------|
| • Netherlands | plantstrengthenener (PPP) – protected crops and field veg's. Re-registration as fungicide |             |
| • UK          | soil improver   | all crops   |
| • Germany     | plantstrengthenener   | all crops   |
| • Belgium     | elicitor (PPP) → fungicide (PPP)  | all crops   |
| • France      | Stimulateur de la vitalité des plantes (PPP)  |             |
|               | crops as NI except root veg's   |             |
| • Italy       | biofungicide (PPP)  | crops as NL |
| • Spain       | Fortificante → excluded after Annex I, pending for PPP                                    |             |
| • Poland      | fertilizer  | all crops   |
| • Greece      | fertilizer  | all crops   |
| • Sweden      | fungicide (PPP)   | crops as NI |
| • Denmark     | fertilizer  | all crops   |

## Products at national level

### **Remedier (Isagro):** *T. asperellum*+*T. gamsii*

- Italy biofungicide (PPP) – protected crops, field veg's, strawberry and turf
- Spain biofungicide (PPP) – protected and field veg's, strawberry

### **Tusal (NBT):** *Trichoderma atroviride* T11 and *T. asperellum* T25

- Spain biofungicide (PPP) – protected crops and field vegetables

### **Agrifutur :** *T. harzianum*, ITEM 908

- Italy Tricover WP and Tricover G: national authorization pending

### Points for discussion

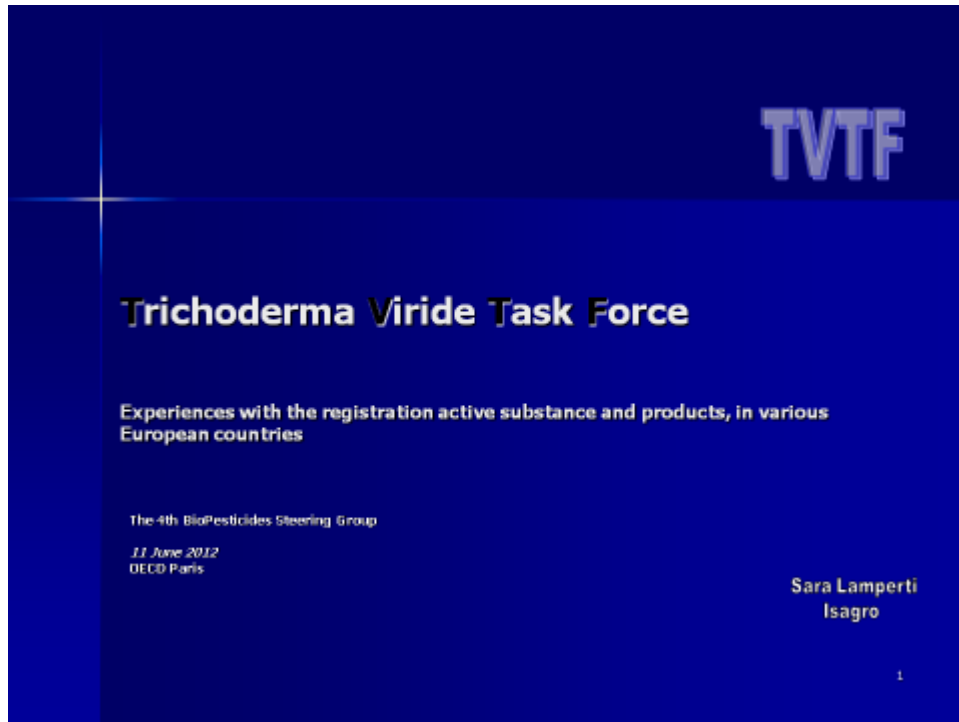
- Many m.o.'s demonstrate Plant Growth Promotion
- Mode of actions: various and variable
- "Old" categorization as fungicide is no longer correct
- Entomopathogenic fungi act also as antagonist, as endophyte, as PGP
- Antagonistic bacteria may have fungicidal, bacterial, insecticidal and PGP effects
- Mycorrhiza show antagonistic, antifungal effects
- Endophytes are common and may be used
- Lack of innovative legislation
- What is claimed ? PGP or pesticidal effects
- EPPO GD (in d'ment) on efficacy does not mention anything on PGP effect, a GD is needed
- New category required for microorganisms

### Next steps

- OECD: To develop a guidance document on Trichoderma (based on lessons learned)
- Protoplast fusion to be officially accepted as a natural process in fungi, and not as a GMO; adaptation of 2001/18/EC
- OECD position in new fertilizer legislation re biostimulants? currently under discussion (SANCO, Agriculture, Industry)
- USA: claim determines PPP or biofertilizer
- National legislation still does not follow EU legislation, very confusing situation
- M.o.'s express various functions: PGP, pesticidal, IR, fertilizing, nutrient uptake facilitating, alleviate biotic and abiotic stress
- Claim should be determinative
- Development of new category or categories of products
- Suggestion: PGP microorganism should not be regarded as PPP
- Innovation in legislation needed

**Task Force II: Experiences with the registration of *Trichoderma viride*: active substance and products, in various European countries**  
**[PPT 10]**

*Sara Lamperti (IsagroRicerca S.r.l., Novara; Italy)*



TVTF

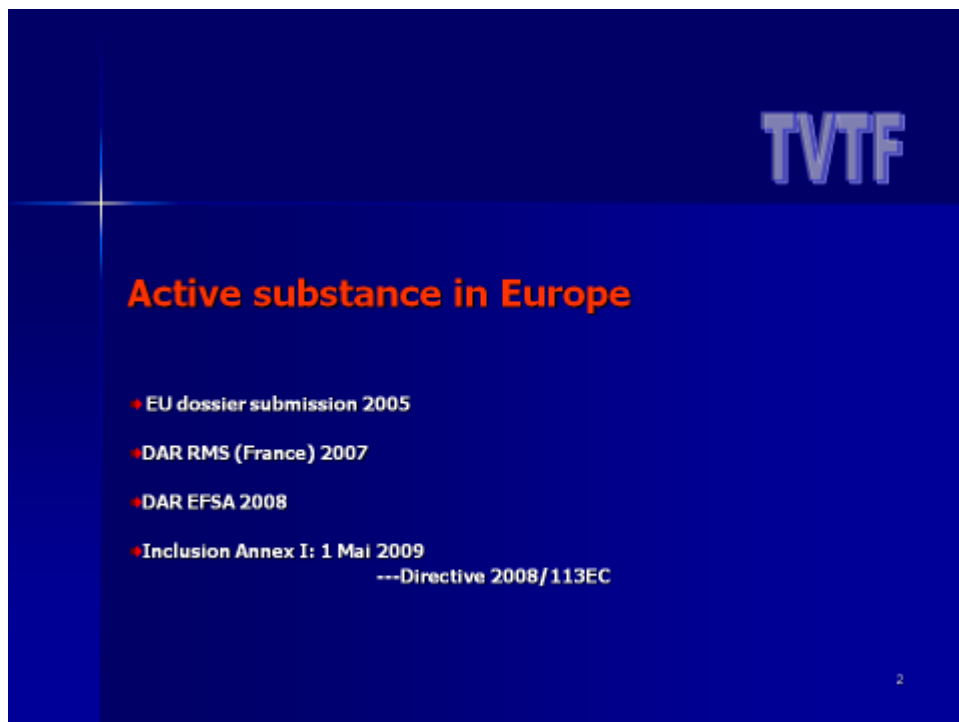
## Trichoderma Viride Task Force

Experiences with the registration active substance and products, in various European countries

The 4th BioPesticides Steering Group  
11 June 2012  
OECD Paris

Sara Lamperti  
Isagro

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TVTF

## Active substance in Europe

- EU dossier submission 2005
- DAR RMS (France) 2007
- DAR EFSA 2008
- Inclusion Annex I: 1 Mai 2009  
---Directive 2008/113EC

2

**TVTF**

## Active substance in Europe

- Commenting period September 2011
- Step 2, 30 April 2012
- Evaluation report EFSA ...2012

3

**TVTF**


## Members TVTF


-  Sevilla, Spain
-  Saint Andiol, France
-  Milan, Italy


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**TVTF**

## Classification change

 Formerly *Trichoderma viride* T-25 → *Trichoderma asperellum* T11

 Formerly *Trichoderma viride* TV-1 → *Trichoderma asperellum* TV1

 Formerly *Trichoderma viride* ICC080 → *Trichoderma gamsii* ICC080

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**TVTF**

## Classification change

The strains are similar with regard to:

- ✓ biology
- ✓ physiology
- ✓ mode of action
- ✓ absence of effects on non-target organisms including humans

The strains can only be distinguished by molecular genetic methods.

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**TVTF**

**Registration as PPP in Europe countries**



**Xedavir** TV1 2,8% - 1 x 107 UFC/g

<i>Country</i>	<i>Target</i>	<i>Crop</i>	<i>Dose</i>
<p><b>Italy</b>                      Registration N°                      13839 of Oct.,                      6th 2008</p>	<p><i>Pythium</i> spp.  <i>Phytophthora</i>  <i>capsici</i>  <i>Rhizoctonia</i>  <i>solanii</i>  <i>Verticillium</i>                      spp.</p>	<p><b>Condimentary Medicinal Aromatical                      and Perfume Plants</b>  <b>Vegetables</b>                      Oilseed rape  <b>Tobacco</b>  <b>Ornamentals</b>                      Flower crops                      Medicinal plants                      Lawns and sports fields</p>	<p><b>3 – 5                      kg/ha                      or                      0,5 – 1                      kg/m<sup>2</sup></b></p>

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**TVTF**

**Registration as PPP in Europe countries**



**Tusal** (*T. asperulum* NBT11 and *T. stramine* NBT25)

<i>Country</i>	<i>Target</i>	<i>Crop</i>	<i>Dose</i>
<p><b>Spain</b>                      Registration N°                      24244 of nov.,                      13th 2008</p>	<p><i>Sclerotinia</i>  <i>Phytophthora</i>  <i>Fusarium</i>  <i>Pythium</i>  <i>Rhizoctonia</i></p>	<p><b>Zucchini</b>  <b>Strawberries</b>  <b>Cucumber</b>  <b>Pepper</b>  <b>Seed of sugar beet</b>  <b>tomato</b></p>	<p><b>3 kg/ha</b></p>

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**TVTF**

**Registration as PPP in Europe countries**



**Remedier** (*T. gamsii* /CC080 and *T. asperellum* /CC012)

Country	Target	Crop	Dose
<p><b>Italy</b> Registration N° of 13199 of March, 7, 2006</p>	<p><i>Rhizoctonia</i> <i>Pythium</i> <i>Sclerotinia</i>, <i>Verticillium</i>, <i>Thielaviopsis</i> <i>b.</i>, <i>Phytophthora</i></p>	<p>Flowers and ornamentals Tomatoes Peppers Lettuce Melons Fennel Artichoke Basil Celery Beans, green beans Zucchini Eggplant Cucumber, Aromatic herbs Strawberries Turf</p>	<p><b>2,5kg/ha</b></p>

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**TVTF**


**Registration as PPP in Europe countries**



**Bioten** (*T. gamsii* /CC080 and *T. asperellum* /CC012)

Country	Target	Crop	Dose
<p><b>Spain</b> Registration N° 25294 of April, 22, 2009</p>	<p><i>Phytophthora</i> <i>a.</i> <i>Verticillium</i> <i>spp.</i> <i>Sclerotinia</i></p>	<p>Eggplant Zucchini Strawberry plants, Lettuce, Melon Olives Pepper Tomato</p>	<p><b>2,5 kg/ha</b></p>

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## Plant Protection Product / Fertilizer

- Induction of disease resistance
  - aspecific
  - specific
- Aspecific resistance>>>Fertilizer/biostimulant
- Label with specific pathogens reported>>>PPP

▶ A clear and unique EU position is needed.

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