

# Non-targeted side effect of microbial fungicides on grapevine: fact or fantasy?

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CONFERENCE, 11 - 12 May 2015, Ljubljana, Slovenia



#### Microbial biocontrol agents

- Commonly regarded as environmentally friendly plant protection products compared to synthetic chemicals
- It does not exclude potential hazards associated with their use
- Toxicological, eco-toxicological studies and evaluation of the fate in the environment (Reg. 1107/2009)









**REGULATION (EC) No 1107/2009** concerning the **placing of plant protection products on the market** and repealing Council Directives 79/117/EEC and 91/414/EEC





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DIRECTIVE 2009/128/EC establishing a framework for Community action to achieve the sustainable use of pesticides



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This Regulation shall apply to substances, including micro-organisms having general or specific action against harmful organisms or on plants, parts of plants or plant products, referred to as 'active substances'



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- Biopesticides (microbial biocontrol agents, botanical extracts) should be registered
- High cost for registration, difficult (and often useless) to study all metabolites, difficult to protect IP for "natural active substances", long process





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- Mutual recognition within the zone (10 ys)



# Low risk substances (15 ys)

#### If it is not

• Carcinogenic, mutagenic,

toxic to reproduction, sensitising

- very toxic or toxic
- explosive
- corrosive
- persistent (half-life in soil is more than 60 days)
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### Basic substances (20 ys)

- Not a substance of concern
- No inherent capacity to cause endocrine disrupting, neurotoxic or immunotoxic effects
- Not predominantly used for plant protection purposes but nevertheless is useful in plant protection either directly or in a product consisting of the substance and a simple diluent
- Not placed on the market as a plant protection product (i.e. milk, molasses, etc.)

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#### Rapporteur Member State assessment reports submitted for the EU peer review of active substances used in plant protection products














#### Development process of a (bio)pesticide









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- Toxicity, pathogenicity and infectivity should be excluded (lab tests on rats)
  - Acute oral administration
  - Acute intratracheal administration
  - Acute intraperitoneal administration





#### Irritation studies

- Rare that irritation (eye, skin) is caused by the microorganism itself
- Co-formulants should be selected to possibly exclude it
- Microorganisms have sensitizing properties in general (operators should wear protections)





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- Genome analysis (to exclude specific pathways) and marker genes expression





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- No accumulation: fast degradation by natural microflora, environmental conditions; no(low) risk of bioaccumulation



### Fate and behavior in the environment

- Fate and behavior in water
- Fate and behavior in air
- Secondary metabolites
- Potential interference with analytical systems for control of the quality of drinking water
- Potential transfer of genetic material to other microorganisms



## Fact or fantasy?

Augmentation (inundation, inoculation)	Native species, but absent or at low levels in the crop (low level of control)	•	mBCA not expected to establish permanently or give autonomous regulation of pathogen Falls back to the natural carrying capacity of the environment over time Some local reproduction (minor) Transient effect
Classical biological control	Exotic biological control agent for the permanent establishment	•	None used with this approach, although classical mBCAs do fall within Reg. 1107/2009
Conservation biological control	Modification of the environment to enhance specific MOs	•	Do not fall within Reg 1107/2009 Involuntarily applied for example by using lignin rich or chitin rich substrates



#### Fate and behavior in water and air

- Most of mBCA cannot grow in water, few of them survive for short time
- Almost all non-spore forming bacteria do not survive in air (desiccation, UV); spore forming bacteria and conidia may survive longer
- Production of secondary metabolites is higher in nutritional substrates than water (very poor production) and almost nil in air



### **European Drinking Water Directive**

- Dir 98/83/EC: microorganisms dangerous to human health, has to be monitored in drinking water (i.e. *Escherichia coli*, Enterococci, *Clostridium perfringens*, *Pseudomonas aeruginosa*, Coliform bacteria)
- The detection can be influenced also by the presence of similar microorganisms (false negative)
- mBCAs are commonly phylogenetically far from these ones (no impact)



### Potential transfer of genetic material

Groups	Туре	Risk
Bacteria	Mutation	Average: 1 mutation per 300 bacterial cells per generation (i.e. antibiotic resistance)
	Natural transformation	Horizontal gene transfer, uptake of extracellular released DNA (plasmid, chromosomal) from mBCA (cannot be excluded. However DNA fast degraded)
	Bacterial conjugation	Relatively frequent in the same species of closely related. Frequent with plasmids
Fungi	Low genome plasticity	HGT never shown in mBCAs; some species parasexual cycle (heterokaryon)



#### Effect on non-target species

- Birds and terrestrial mammals
- Aquatic organisms
- Bee and other non target arthropods
- Earthworms
- Soil-microorganisms
- Terrestrial plants



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#### **HOST SPECIFICITY**



#### **Read-across**

Extrapolation of data between species or strains to aid risk assessment

Depends on:

- Phylogeny
- Phenotype
  - Physiology or morphology
  - Behaviour
  - Emission of substances
    - Class of substance
    - Chemical structure
    - Mode of action

Source: EFSA supporting publication 2013:EN-518





- Infectivity and pathogenicity
- Production of metabolites/toxins
- Toxicity endpoints and adverse effects :
  - Type of adverse effects
  - Type of organism threatened by the adverse effects
  - Dose-effect relationships (including the noobserved-adverse-effect levels), mechanisms underlying the adverse effects
    Source: EFSA supporting publication 2013:EN-518



#### Study cases

#### TRICHODERMA SPP., TRICHODERMA ATROVIRIDE SC1 LYSOBACTER CAPSICI AZ78





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- The term "secondary metabolite" includes a **heterogeneous group** of chemically different natural compounds possibly **related to survival functions** for the producing organism, such as **competition** against other micro- and macroorganisms, **symbiosis**, metal transport, differentiation, etc.



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- The term "secondary metabolite" includes a **heterogeneous group** of chemically different natural compounds possibly **related to survival functions** for the producing organism, such as **competition** against other micro- and macroorganisms, **symbiosis**, metal transport, differentiation, etc.
- Production may vary according substrate





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Trichoderma spp. antibiotics

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- **Peptaibols** (linear oligopeptides of 12–22 amino acids )
- Only four species not closely related to the ones used in biocontrol (*T.brevicompactum, T. arundinaceum, T. turrialbense,* and *T. protrudens*) produce **trichothecenes**


#### Two different mechanisms of action

From Vinale et al. 2008

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#### Two different mechanisms of action

• Low molecular weight, non-polar, volatile compounds (i.e. 6PP): high concentration of antibiotics in the soil environment and relatively long distance range of influence on the microbial community



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- Low molecular weight, non-polar, volatile compounds (i.e. 6PP): high concentration of antibiotics in the soil environment and relatively long distance range of influence on the microbial community
- A short distance effect may be due to the polar antibiotics and peptaibols acting in close proximity to the producing hyphae

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 Detection of other fungi and grow tropically towards them (remote sensing)





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- Attach to the host and coil around it and form appressoria





- Detection of other fungi and grow tropically towards them (remote sensing)
- Attach to the host and coil around it and form appressoria
- Fungitoxic cell-wall-degrading enzymes, and peptaibol antibiotics are produced





 Produces high molecular weight compounds that reach the host
Low molecular weight-degradation products are released from the host cell walls reach the mycoparasite and activate the mycoparasitic gene expression cascade

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# Lysobacter: metabolites of concern

- Lysobacter genus: no production of any metabolite that can be harmful for humans or that can be of concern for the environment
- L. capsici AZ78 genome (sequenced): absence of genes involved in the biosynthesis of metabolites that are of concern for human health
- No extra chromosomal genetic element
- Genome analysis: most robust tool





# Natural occurrence of Trichoderma

- 10-10<sup>2</sup> colony forming units /g soil and common wood/leaf litter degrader
- Following application of *T. atroviride* SC1, increased presence
- After one year, concentrations at natural background levels





### SC1: impact on soil microorganisms

Cluster analysis of ARISA profiles bacterial (a) and fungal (B) community structures following introduction of *T. atroviride* SC1 Seasonal variation

- higher than with SC1
- SC1: only transient effect





# Natural occurrence of Lysobacter

- Genus Lysobacter: ubiquitous bacteria and common inhabitants of soil and plants
- Also river sediments, municipal solid waste, biofouling materials, hydrothermal vents, mud flows, upflow anaerobic sludge blanket reactor, beach sand soils, geothermal soil and field, Antarctic aerosol and freshwater, deep seas sponge, arbuscular mycorrhizal fungi, seal feces, penguin feathers, salamander skin, brook charr skin, earthworms, nematodes....



# Persistence of *L. capsici* AZ78 on phyllosphere

• Limited persistence (to 0 in few weeks)



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# AZ78: impact of phyllosphere microorganisms

Metagenomic approach (leaf treatments, grapevine)







#### Fact or fantasy: conclusions



- Smart selection of active microorganism strain (identity, host range, and ecological characteristics)
- Genome sequencing and marker gene expression, targeted HPLC, LC-MS (metabolites, (eco)toxicity, mechanism of action)
- Confirmatory tests on non-targets
- Metagenome analysis of target environments



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Thank you for attention!



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