REBECA Workshop

Potential Risks of Microbials

New insights into risk evaluation and registration of microbial BCAs in Europe

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FUNGAL METABOLITES

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DATA REQUIREMENTS FOR THE REGISTRATION OF A MICROBIAL BCA (OECD, 1996)

- Identity
- Physical, chemical and biological properties
- Function, mode of action and handling
- Manufacturing, quality control and analytical methods
- Residues
- Efficacy
- Toxicology
- Ecotoxicology
- Fate and behaviour in the environment

INFORMATION ON THE PRODUCTION OF METABOLITES (directive 91/414/EEC)

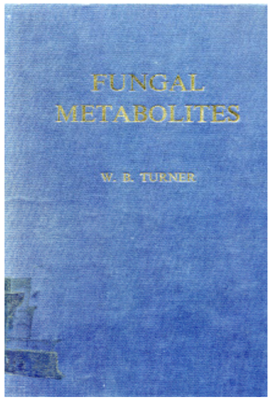
"Where the microbial species of a strain subject to the application is known to produce metabolites […] that have ***undesirable effects*** on human health and/or the environment and […] are produced in significant quantities during or after application, the ***nature and structure*** of this substance, […] its stability, its mode of action […] ***effect on humans, animals or other non-target species*** shall be indicated".

FUNGAL SECONDARY METABOLITES (FSM): WHAT ARE THEY AND WHY ARE THEY PRODUCED?

FSM are relatively ***small, complex organic compounds*** that originate as derivatives from various intermediates in the primary metabolism.

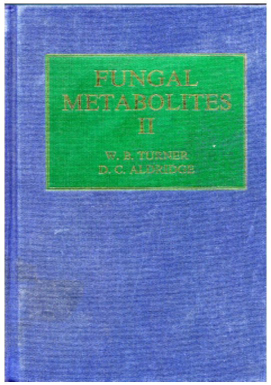
FSM ***play no obvious role in the metabolism of the organism***, and typically:

- They occur optimally after a phase of balanced growth and often (but not always) in association with morphologic changes (e.g. sporulation)
- The production of particular secondary metabolites is usually restricted to a small number of species and may be ***species, or even strain, specific***
- It has not generally been possible to rationalize the ***biological function of FSM***, although some are very active against microorganisms (antibiotics), plants (phytotoxins) or animals (mycotoxins)



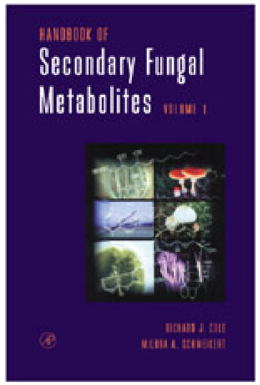
Vol. I, 1971

828 compounds



Vol. II, 1983

About 2000 new compounds



3 Vol.s, 2003

SELECTED METABOLITES OF SOME IMPORTANT FUNGAL BCAs

Fungal BCA	Main Target	Metabolites produced *in vitro* and/or *in vivo*
Trichoderma spp.	Fungi	Peptaibols (23 families, 182 forms), trichothecenes, α-pyrones, isonitriles
Gliocladium spp.	Fungi	Gliotoxin, gliovirin, viridin, viridiol, glisoprenins, antiamoebin, heptelidic acid
Fusarium spp.	Fungi, insects and weeds	Trichothecenes, beauvericin, moniliformin, fusaric acid, naphthazarins
Metharizium anisopliae	Insects	Destruxins (> 27 types), swainsinone, cytochalasin C
Beauveria bassiana	Insects	Bassianin, beauvericin, bassianolide, beauverolides, tenellin
Beauveria brongniartii	Insects	Oosporein
Verticillium lecanii	Insects	Dipcolonic acid, hydroxycarboxylic acid, cyclosporin
Colletotrichum spp.	Weeds	Colletotrichin
Stagonospora spp	Weeds	Elsinochrome A, cercosporin, leptosphaerodione

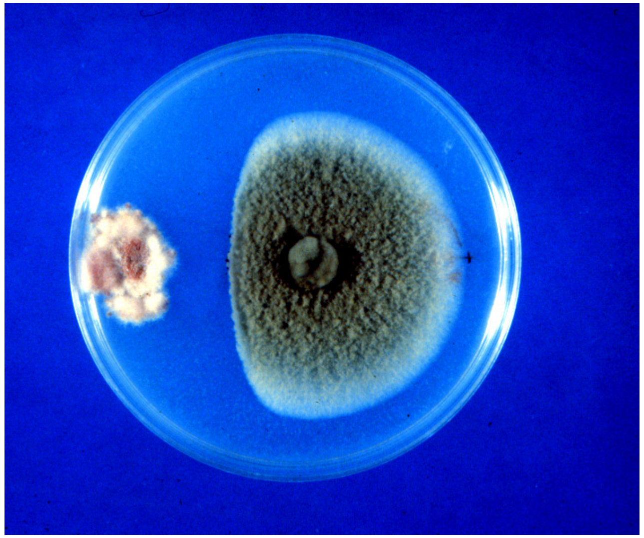
PHYSIOLOGICAL AND ECOLOGICAL SIGNIFICANCE OF FSM PRODUCED BY BCAs

- Antibiotics, siderophores → Antagonists
- Phytotoxins → Mycoherbicides
- Toxins, antifeedant substances → Entomopathogens

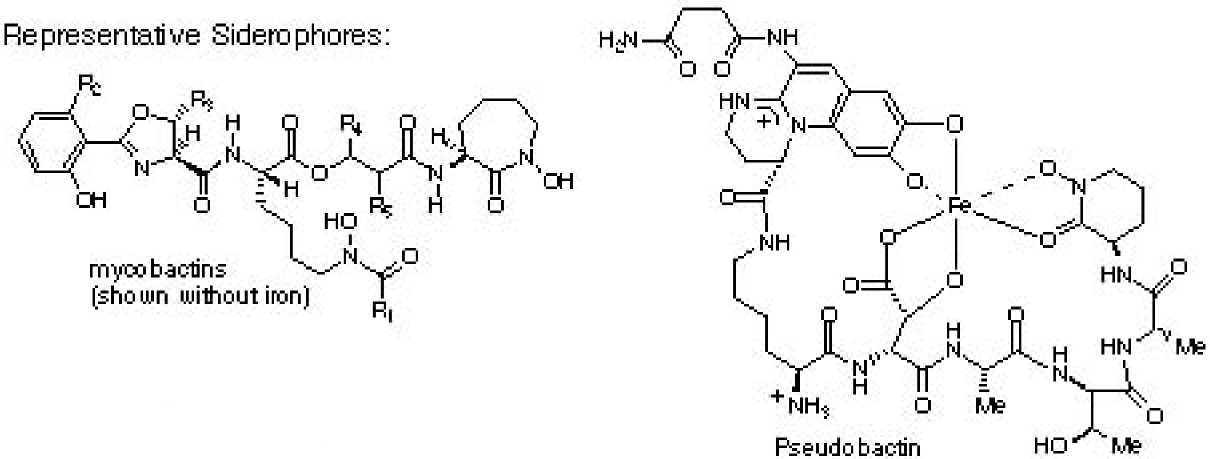
ANTIBIOTICS AND SIDEROPHORES

- **Target effects**: inhibition or disruption of the growth, development or behavior of plant pathogenic microorganisms. They enable the BCA to compete for nutrients and space

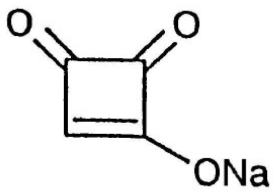
- **Non-target effects**: toxicity to nontarget microorganisms naturally present in soil or other habitats where the BCA is introduced (VAM fungi, saprophytic fungi, cultivated mushrooms, symbiontic bacteria)



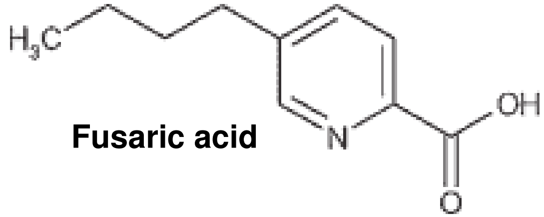
Representative Siderophores:



Moniliformin



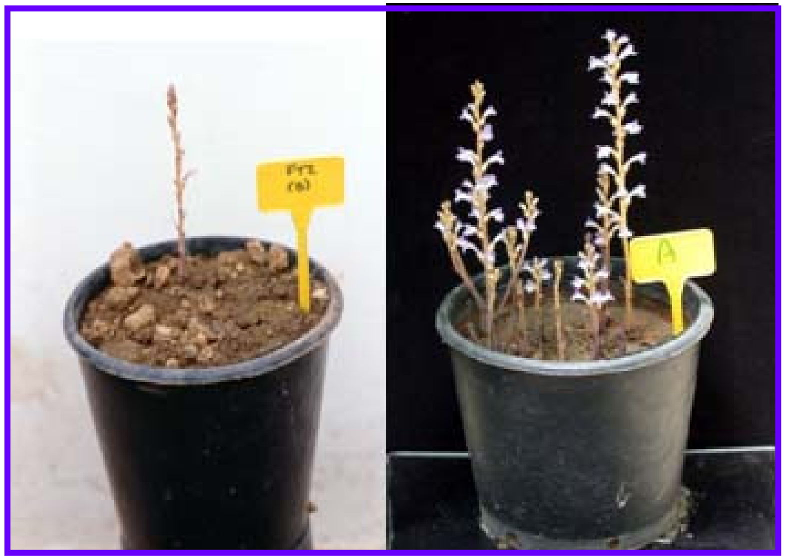
PHYTOTOXINS

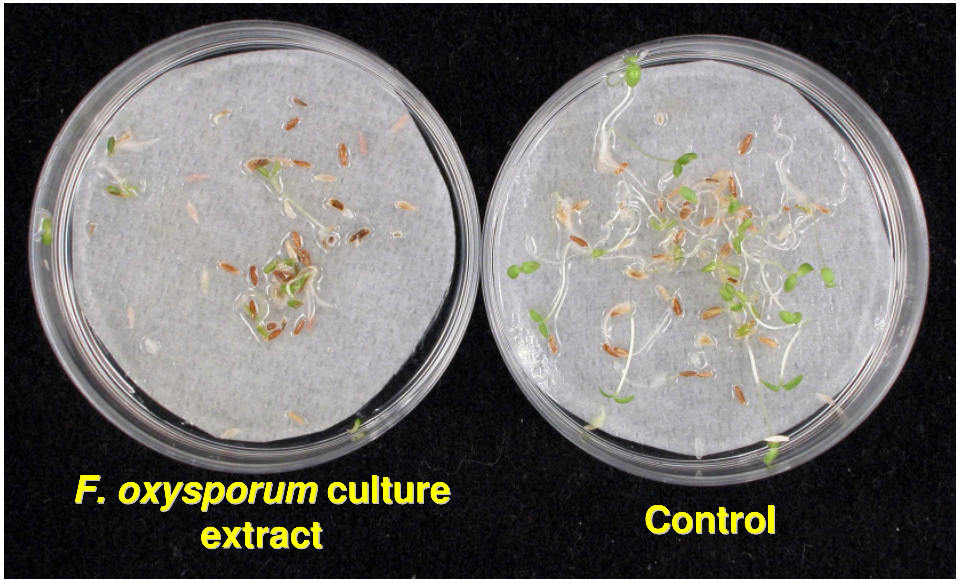


Fusaric acid

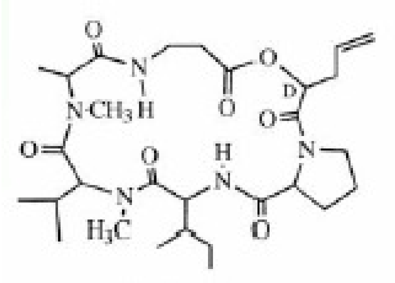
- **Target effects**: contribute to phytopathogenicity or virulence of mycoherbicides to target weeds by weakening the plant defence response

- **Non-target effects**: inhibition of germination and/or growth of non-target plants, including crop plants





Destruxin



ENTOMOTOXINS

- **Target effects**: pathogenicity determinants of entomogenous fungi (e.g. kill or weaken host by suppressing host immune system). Antifeedant/repellent properties that prevent or reduce pest damages
- **Non-target effects**: Toxicity to birds that feed with infected cadavers. Possible adverse effects on beneficial arthropods (e.g. *Collembola*)

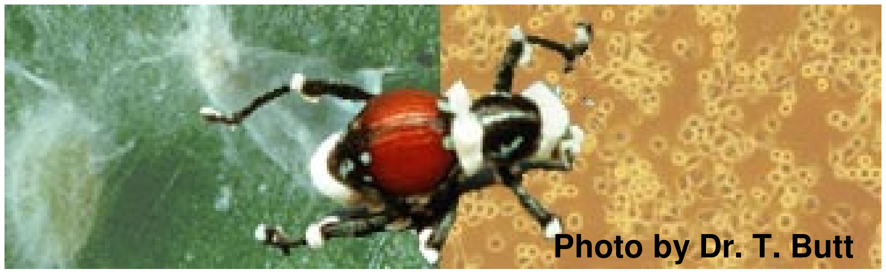
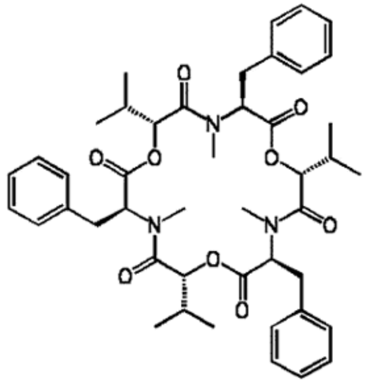


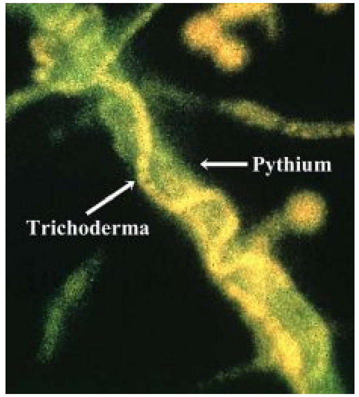
Photo by Dr. T. Butt

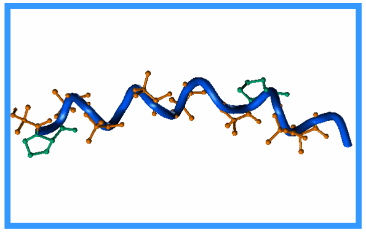


BEAUVERICIN

EXAMPLES OF POTENTIAL ADVERSE EFFECTS OF SELECTED FSM TO NON-TARGET ORGANISMS

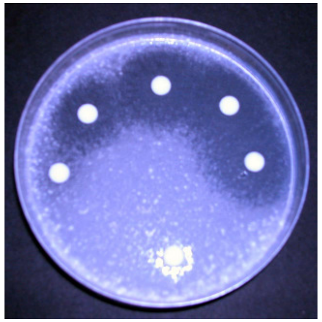
1. Alamethicin** (member of the peptaibol class of metabolites), produced by **Trichoderma viride




Alamethicin

Possess membrane-modifying properties

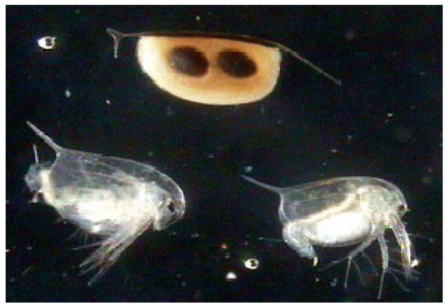
Exhibit antimicrobial activity (*vs*. fungi and Gram- bacteria)



Zootoxic in the invertebrate models *Artemia salina* (LD_{50} = 2 μM at 36h) and *Daphnia magna* (LD_{50} = 1 μM at 36h)

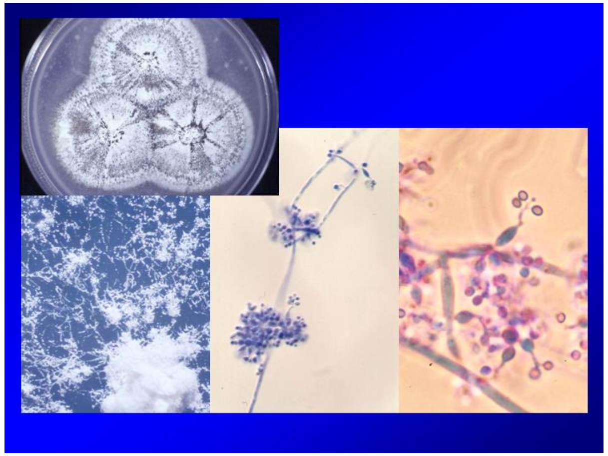
Cytotoxic to human and insect cell lines (CC_{50} 2.5 – 13 μM at 4h)

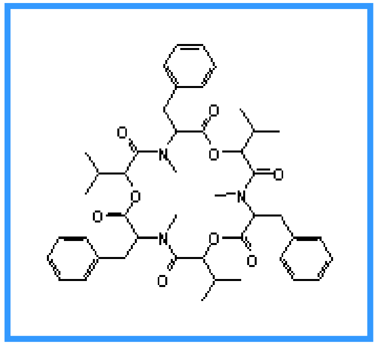
Acute toxicity to mice, oral administration, LD50 = 80 mg/kg



EXAMPLES OF POTENTIAL ADVERSE EFFECTS OF SELECTED FSM TO NON-TARGET ORGANISMS

2. Beauvericin (*Beauveria* spp., *Fusarium* spp.)





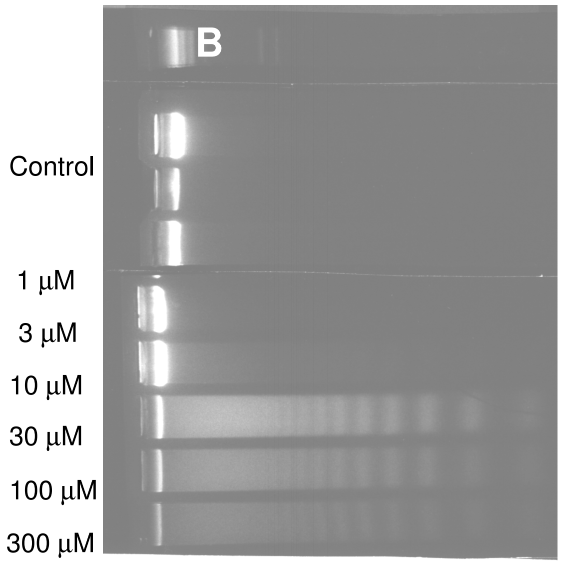
Beauvericin

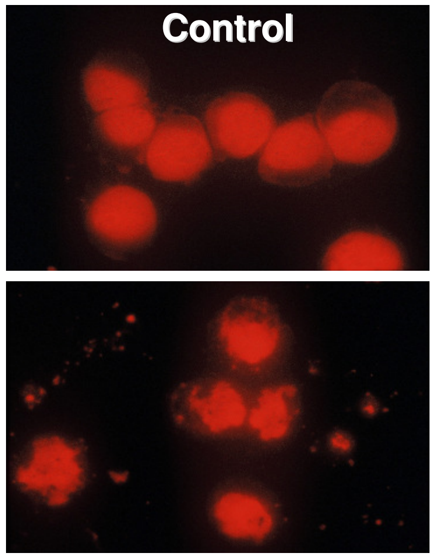
- Cytotoxic to human and insect cell lines (CC_{50} 20 - 40 µM at 4h)

- Pro-apoptotic effects at concentration 30 µM (2 h) or higher

- Acute toxicity to mice, intraperitoneally, LD50 = 1 - 1.35 mg/kg

- Zootoxic to *Artemia salina* (LD_{50} = 3.6 µM)





RISK ASSESSMENT OF FMS

Risk is the *probability of occurrence of a particular adverse nontarget effect*. Risk is a combination of 2 factors:

- **Hazard**: inherent property of an agent or situation capable of having adverse effects on something. Hence, the substance, agent, source of energy, or situation having that property
- **Exposure**: Concentration, amount, or intensity of a particular agent that reaches a target system. It is usually expressed in numerical terms of substance concentration, duration, frequency, and intensity

HAZARD IDENTIFICATION AND CHARACTERIZATION

- Characterization of the producing species and the strain - Genetic potential of the strain (toxigenicity)
- Factors that have an influence on the biosynthesis of FSM
  - ✓ Nutritional conditions (C source, C/N ratio)
  - ✓ Environmental conditions (T, RH, light, etc.)
- Identification of the relevant metabolites (relevant metabolites are metabolites that are of concern for human or animal health and/or the environment, 2001/36/EU)
- Toxicological characterization of the relevant metabolites by methods of in vitro toxicology and animal-based toxicology

EXPOSURE ASSESSMENT

Factors that have an influence on the occurrence of FSM in the environment

- Host range and ecological behaviour
- Application method (classical, inoculative, inondative)
- Population dynamics
- Persistence and dispersal (residues: viable micro-organisms and substances produced in significant quantities by these micro-organisms which persist after the disappearance of the micro-organisms and are of concern for human or animal health and/or the environment, 2001/36/EU), affected by:
    - <u>Climatic factors</u>: inactivated by UV radiation, heat, washed by rain
    - <u>Edaphic factors</u>: adsorbed on soil particles or denaturated by phenolics, acids
    - <u>Biotic factors</u>: broken down or taken up by plants and/or soil micro-organisms

RISK ASSESSMENT OF FUNGAL BCAs' METABOLITES: THE CHEMICAL APPROACH

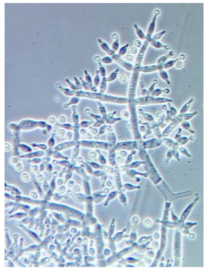
- Identification of the toxic metabolites produced by the species the BCA belongs to
- Analysis of the BCA strain for <u>known</u> toxic metabolites
- Toxicological and ecotoxicological assessments of pure metabolites
- Fate and behaviour of metabolites in the environment

HURDLES AND PITFALLS IN USING THE CHEMICAL APPROACH
1. Identification of the toxic metabolites *produced by the species the BCA belongs to*

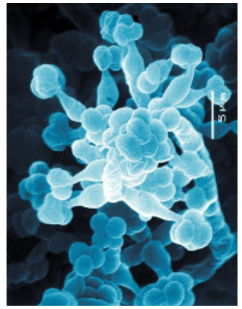
- Our knowledge of the array of bioactive metabolites produced by fungal species is still largely incomplete
- Unknown and potentially toxic metabolite(s) might be produced by one particular BCA strain

HURDLES AND PITFALLS IN USING THE CHEMICAL APPROACH
*2. Analysis of the BCA strain for **known toxic metabolites***

- Production of metabolites may vary greatly depending on growth conditions
- Reliable and accurate analytical methods not always available
- Setting and validation of analytical methods and purification of standards is extremely costly and time-consuming (nor economically feasible for all the known metabolites)
- Reference standards (pure compounds) available only for very few metabolites

Metabolites of *T. harzianum*

Class	No. of compounds	Compounds
Peptaibols	5	Harzianins, trichokindins, trichororzins, trichozianines, trichorzins
Antraquinone pigments	2	1-hydroxy-3-metyl antraquinone, 1,8-dihydroxy-3-metyl antraquinone
Oxygen heterocyclic compounds	5	Harzianopyridione, harzianolide, dehydro-harzianolide, harzianic acid, trichoharzin
Pyrones	2	6-pentyl-α-pyrone, 6-pent-1-enyl-α-pyrone
Octaketides	6	Koninginin A, B, D, E, hydroxy koninginin B, seco koninginin
Cyclonerodiols	1	Cyclonerodiol
Trichothecenes	2	Harzianum A, harziandione
Isocyano derivatives	4	Isonitrin A, isonitrin D, homothallin II, MR304A
Others	3	Uracil, melanoxadin, ceramide
TOTAL	**30**	

HURDLES AND PITFALLS IN USING THE CHEMICAL APPROACH

***3. Toxicity and ecotoxicity assessments** of pure metabolites*

- Possible synergistic action of two or more metabolites produced contextually is not taken into consideration

HURDLES AND PITFALLS IN USING THE CHEMICAL APPROACH

4. Fate** and behaviour of metabolites **in the environment

Extraction from organic matrices with satisfactory recoveries is very difficult

- adsorbed on soil particles and by organic matter
- Metabolized by soil microrganisms and by plants to either less or more toxic products

RAFBCA

Fifth Framework Programme

Risk assessment of biological control agents

Dr. Tariq Butt, University of Wales Swansea, UK, Coordinator

RAFBCA Objectives

Do BCAs' metabolites enter the food chain and pose a risk to human health?

1. Identify the major metabolites secreted by fungal BCAs (i.e. those most likely to enter the food chain)
2. Develop the methods and tools to monitor metabolites and fungal BCAs in the environment
3. Develop *in vitro* toxicity assays such as sensitive cell lines (i.e. biosensors) to detect selected metabolites
4. Determine the role and mode of action of metabolites to identify target sites and potential risks
5. Monitor major metabolites in the environment to see if they enter the food chain. Evaluate the risks they pose to human and animal health, if any.

RAFBCA

Risk assessment of biological control agents

Fifth Framework Programme

Dr. Tariq Butt, University of Wales Swansea, UK, Coordinator

✓RAFBCA took 3 years in method development and validation of extraction procedures for limited number of metabolites, food crops, and target hosts

✓Attention was focused on major metabolites but what about the other metabolites?

✓It would be impossible to determine risk of every single metabolite.

- Costly in resources
- Takes many years
- Requires a high degree of expertise

CONCLUSIONS

- ✓If the chemical approach is used for risk assessment of fungal BCAs' metabolites, is very difficult that any BCA can make it out of the laboratory and into the market, and biological control is doomed to remain an exercise for scientists

- ✓There is the need of a revision of the data requirements for the registration of biological pesticides that take into account the unique nature of these pesticides and address safety issues without at the same time unduly hamper the development and implementation of microbial control

- ✓"…the registration requirements for biocontrol fungi must remain flexible and address the hazards of the specific candidate in question and its proposed use on a case-by-case basis" (Goettel M.S. *et al*., 2001)