Objectives:

Identify the metabolites produced by fungal biological control agents (BCAs) and establish whether they enter the food chain and if they pose a risk to human and animal health.

More specifically to:

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 (species-specific quantitative PCR, NASBA, species-specific microarrays) 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, 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.

Results and Milestones:

WP1. Identification of major metabolites secreted by Fungal BCAs

The RAFBCA team have accomplished the milestones for this workpackage. Briefly, the RAFBCA team have:

- Identified virulent strains of fungal BCAs
- Identified the major metabolites secreted by these fungal strains
- Generated data that provides a better understanding of the distribution and regulation of selected major metabolites
- Established inter- and intra-specific variation in the production of these metabolites
- Generated data on the type and quantity of each major metabolite.

WP2. (Development of tools and methodologies to monitor metabolites and fungal BCAs in the environment) and WP3 (Identifying Sensitive Cell Lines (i.e. biosensors) to detect selective metabolites) These two workpackages were being developed concomitantly because biosensors may be used in high throughput microtitre plate assays. RAFBCA have accomplished the following:

- The MTT test was shown to be a convenient microplate bioassay to monitor fungal BCA metabolites like destruxins (dtx) in the environment. Calcein AM staining also showed considerable promise.
- Of all the cell lines screened, the *Sf-9* cell line (derived from the epithelium of the *Spodoptera frugiperda* ovaries) was shown to be the most sensitive to the widest range of fungal metabolites. Identification of a sensitive cell line was one of the **deliverables** of WP2.
- The ciliate protozoa, *Tetrahymena pyriformis* and *Paramecium caudatum,* were shown to be highly sensitive to fungal metabolites and possible “environmental biosensors”. For example, *T. pyriformis* was far more sensitive to elsinochrome A, a major metabolite of *Stagonospora* (mycoherbicide) than the

bacterial species used in microtitre assays. *Paramecium caudatum* was sensitive to 1-5 µM oosporein, a major metabolite of the insect pathogen, *Beauveria brongniartii*.

- Species-specific primers for *M. anisopliae* were constructed by sequencing and analyzing the complete mtDNA genome (25.5 kb) and rDNA nuclear repeat (8.2 kb) of this fungus. This will help monitor the fungus in the field. A similar approach was completed for *V. lecanii* and *B. brongniartii*.
- The entire *tri5* gene has been obtained and sequenced from *T. harzianum* ATCC90237 (a recognized producer of the trichothecene harzianum A). It shows approx. 65% similarity to *tri*5 gene sequences from other fungal species.

WP4. Determine the mode of action of metabolites to identify target sites and potential risks

The RAFBCA team have made considerable progress in this area. Some key findings are listed below.

- An apoptotic effect of dtx A and dtx E on *Sf-9* cells *in situ* at the single cell level was confirmed using the DeadEnd TUNEL assay.
- Dtx was shown to activate caspases in *Sf-9* cells using the Polycaspases FLICA assay and the CaspACE^{TM} kit designed for mammalian cells.
- Beauvericin-induced apoptosis was observed in three mammalian cell lines of myeloid origin (*i.e*. the rat mast cell line RBL-1, the human promyelocytic HL60 cells and the human monocytic U937 cells). This was shown by agarose gel electrophoresis of DNA degradation, fluorescence microscopy (using the DNA stain - propidium iodide) and the caspase-3 enzyme assay.
- Dtxs were shown to act on calcium channels and induce calcium fluxes in *Sf-9* cells which, subsequently, affected the integrity of cytoskeletal elements. The antagonist $CdCl_2$ partially inhibited the effect of dtx A on the depolymerisation of actin filaments whereas nifedipine did not.
- Dtx A was shown for the first time that it can activate adenylate cyclase leading to an increase in intracellular camp. However, dtxs were found to have no affect on tyrosine kinase activity.
- The Ames test showed that oosporein and the crude extracts of *V. lecanii* (Mycotal and Vertalec) and *M. anisopliae* (V245 and V275) were not mutagenic. This supports findings of the Vitotox genotoxicity assay.

WP5. Monitor major metabolites in the environment to see if the enter the food chain

The RAFBCA team have briefly accomplished the following:

- Developed and validated methods for extraction and detection of metabolites in plants and insects.
- Conducted trials under commercial/semi-commercial conditions to determine the fate of fungal BCAs and their metabolites in the food chain (e.g. lettuce, cucumber, tomato) and plant growing media.
- Results showed that the selected BCA metabolites evaluated did not enter the food chain nor pose a risk to humans and the environment. The metabolites were absent or if present then the levels were extremely low (i.e. concentrations were too low to pose a risk).
- Established that dtxs A, B and E are degraded in cadavers of *Tenebrio molitor* and *Galleria mellonella* infected with *M. anisopliae* with dtx E being the least stable compound. Due to their rapid degradation, it is unlikely that these compounds will contaminate the soil or groundwater.

- Environmental conditions (substrate, temperature, humidity and pH) were shown to influence the stability of fungal metabolites. Some examples are listed below:

  1. Dtxs are stable on inert glass surfaces but degrade on plant surfaces. Only 40 % dtx A and 20 % dtx B was recovered from tomato leaves, 14 days post-incubation under glasshouse conditions.
  2. Dtxs are denatured by boiling while oosporein has a half-life of 0.3 days at 53 °C.
  3. Dtx E is unstable at low RH and cannot be recovered after 4 weeks at 8% RH.
  4. Oosporein degrades quickly under moderately alkaline conditions (half-life is 12 days at 23°C, pH 8) but is more stable under moderately acidic conditions (half life is 74 days at 23 °C, pH 6).
  5. Elsinochrome A is not degraded by boiling or cooking but is highly unstable when exposed to daylight. It has a half-life of 14 h on sunny days (8 - 10 hours of sunshine/day) and of 99 h when exposed to daylight on cloudy days (0 - 2 hours of sunshine/day). Photo-degraded elsinochrome A was not toxic to the highly sensitive test organism *Trichoderma atroviride* P1.