First Progress Report

1. OBJECTIVES

The aims of the RTD project FAIR6-CT98-4105 are: (1) to control scarabs and weevils with virulent, ecologically competent strains of insect-pathogenic fungi, (2) to improve production and formulation technologies, (3) to develop biochemical methods to monitor fungal virulence and nutritional (carbon) requirements, (4) use molecular techniques to characterise strains for the monitoring of the pathogen in the field (spatial-temporal distribution, genetical stability, interactions with autochthonous, conspecific strains) and for the detection of instability factors (e.g. transposons, mycoviruses), (5) to test new application systems for effective targeting of the pathogen, (6) to study the impact of the pathogens on target and non-target insects, (7) to conduct field trials to demonstrate/evaluate the efficacy of the fungal biological control agents and (8) to address some of the criteria for the registration of insect pathogenic fungi.

2. DESCRIPTION OF WORK

During the first six months of the project the BIPESCO-team started to perform bioassays against scarab (Melolontha melolontha) and weevil larvae (Strophosoma and Otiorhynchus) using isolates provide by the partners. The aim of these assays was to identify the most efficacious strains, that is those which are virulent (kill in as short as time as possible) and are ecologically fit (persist well and retain virulence under field conditions). Protocols were developed for rearing of pest species to facilitate more extensive assays. Bioassay procedures were standardised and included: (1) the dipping method Ü direct application technique (2) the indirect application technique whereby insects were exposed to sporulating culture on agar or barley kernels and (3) soil application of inoculum to defined volume of infested soil. The protocols are in the process of being refined.

Physiological studies were conducted to learn more about attenuation of virulence. Inoculum (filamentous mycelium and conidia) from successive subcultures of different Beauveria and Metarhizium strains were assayed against target species to identify strains which lose virulence quickly. Enzyme profiles have been prepared for some strains and for some subcultures partly for strain characterisation and partly to identify pathogenicity-related enzymes. In addition, analyses have been and made of secondary metabolites which include suspected pathogenicity determinants. Particular attention focused on destruxins, efrapeptins and oosporein.

Concomitantly, small scale fermentation processes were run to optimise the production of fungal biological control agents (BCAs) in liquid- and solid state bioreactors. New formulation techniques have been tested and are in progress to increase the shelf life of BCAs. One technique is to compress the formulation components (glidant, lubricant and anti-adhesive) and fungal conidia of Metarhizium anisopliae directly after mixing.

The knowledge from RFLPs of mtDNA, protease-gene differences and various amplified rDNA regions (18S, ITS-5.8S-ITS, 28S) which were developed are used to optimize detection conditions at the species and strain level of Beauveria and Metarhizium. Knowledge of the spatial-temporal distribution of inoculum is essential to our understanding of how epizootics are initiated. For this reason, studies on the persistence of B. brongniartii and M. anisopliae in

the soil are carried out at sites where these pathogens have already been used for scarab and weevil control for many years. In spring soil samples were taken from treated and untreated (control) plots to monitor the population dynamics of the pathogens and to provide information on the persistence of the pathogen by quantifying the degradation (shelf life) and recovery of the pathogen.

State of Progress

Quantal mortality data and time-response studies helped distinguish between insects killed by the BCA from those killed due to other reasons. The efficacy of Beauveria brongniartii isolates against Melolontha melolontha larvae were established. LC50 studies showed that mortality was dose-related for M. :anisopliae and B. brongniartii irrespective of the method of inoculation. However, the latter result was obtained for treated soils using the "Galleria baitî method. This method will reveal more about the effect of environmental (edaphic, biotic, climatic) factors on the ecological fitness of virulent strains and frequency of application. Subculturing did affect the phenotype and virulence of Beauveria brongniartii and Metarhizium anisopliae. Attenuation of virulence was manifest in two different ways; (1) a gradual decline in virulence and (2) an initial decline and then a recovery. Most studies to date report a gradual or rapid decline in virulence with recovery only being achieved by passaging through an insect. Bioassays alone did not reveal the full variation in expression of attenuation but together with microscopy studies a considerable amount was learnt about the attributes of virulent isolates. For example, subtle differences were noted in the adhesion and the germination pattern of M. anisopliae conidia from different subcultures. This affected the overall mortality and LT50 value. This, together with changes in the Pr1 induction response in selective medium suggest that subculturing induces physiological changes in the fungus. Recently developed primers can distinguish between species but specific primers will be designed to check for similarities/differences for all isolates in the BIPESCO collection. Field trials were conducted using different isolates of B. brongniartii against second/third-instar larvae of Melolontha melolontha. First results show, that 77-94% of larvae from northern Italy (Auer, South Tyrol) succumbed to mycosis with an average survival time (AST) of 22.9-40.1 days. Infections in larvae from south-western Switzerland (Bramois, Valais) were 28-72% and the ASTs varied between 34.7-56.4 days. Differences in susceptibility between the two host populations may be explained by the historical ages of the two populations and the presence of B. brongniartii resulting in a coevolution of tolerance between the host and pathogen. The Italian population is occasionally infected by B. brongniartii whereas the Swiss population, has experienced periodic epizootics over a 50 year period. Coevolution between B. brongniartii and M. melolontha from Switzerland may explain the apparent resistance of the host towards this pathogen in laboratory assays.

3. ACHIEVEMENTS

The first milestone, exchange of cultures, has been achieved. The BIPESCO collection consists of a wide range of strains the majority of which were isolated from infected larval stages of scarabs, weevils or from the soil. A large number of bioassays were carried out because of the large number of test insects collected by the partners. Eight strains of both B. brongniartii and M. anisopliae will be selected for ongoing research activities within the next six months. Strains are already distributed to all partners and stored in their culture collections.

4. FUTURE ACTIONS

Protocols for maintaining insect cultures to provide larvae for bioassays needs to be developed/refined. Data on attributes of fungal strains (virulence, specificity for scarab species and weevils) needs to be completed. Stability of strains and secretion of metabolites by strains of commercial potential needs to be determined. Attention will focus on adhesion and the production of oosporein, destruxins and Pr1 which are considered to be linked with pathogenicity. Molecular studies will be initiated to elucidate the molecular basis for the fluctuation of virulence in selected Metarhizium strains. A range of media which stabilise virulence will have been identified within the next six to nine months and an analysis made of growth, spore production on media with known C:N content. The industrial partners will run several small-scale liquid- and solid state fermentation studies to build the basis for the up-scaling of the production process. Various parameters have to be monitored in both small and medium scale fermentation systems including fungal growth (pH, pO2, glucose consumption), inoculum yield and shelf life. Particular effort will be made in the development of liquid and granular formulations with enhance shelf life, sporulation and growth after application. The sequencing of the entire rDNA gene complex of M. anisopliae was set as a target during the first six months. At present almost 70% of the entire rDNA gene-complex region has been sequenced in both directions. It is anticipated that the entire sequence will be available soon, and that by that time specific primers will be designed to check for similarities/differences between isolates in the BIPESCO collection. Comparisons of the rDNA sequences available today are made with PCgene and data banks. These already allow the detection of some differences at species level which will be used in the future. The evaluation of survival and distribution of B. brongniartii at sites where the fungus was applied periodically will be intensified in the next months, using additional test sites also in Italy, Austria and Switzerland. Periodically, the sites will be evaluated regarding the quantity and virulence of B. brongniartii-spores, infection of Melolontha-larvae and detectable damage of crops. Additional Beauveria and Metarhizium strains will be isolated by the Galleria bait method and through the use of selective media. The impact of entomogenous fungi on target and non-target insects and on the behaviour of pest insects in soil where M. anisopliae was applied have been initiated and will be continued. Strains of M. anisopliae var. anisopliae, M. flavoviride, B. brongniartii, B. bassiana, P. farinosus and of P. fumosoroseus have being tested against the weevils S. melanogrammum and O. singularis since July 1999 and field trials in Christmas tree plantations are scheduled in the near future.