

ABSTRACT

The aim of the BIPESCO project was to study and develop entomogenous fungi for the control of subterranean insect pests like scarabs and weevils. Leading experts from seven different European countries including four industrial partners participated in this multidisciplinary, multifaceted project. Particular attention focussed on the widespread, soil-borne pathogens, *Beauveria brongniartii* and *Metarhizium anisopliae*. Strains of these fungi were developed for use in integrated pest management programmes to help replace or reduce the input of chemical pesticides in European agriculture, forestry and horticulture in accord with the reformed common agriculture policy guidelines. The specific objectives of this project were to (1) control scarabs and weevils with virulent, ecologically competent strains of insect-pathogenic fungi, (2) improve production and formulation technologies, (3) develop biochemical methods to monitor fungal virulence and nutritional (carbon) requirements, (4) use molecular techniques to characterise strains to monitor the pathogen in the field (spatial-temporal distribution, genetic stability, interactions with autochthonous, conspecific strains) and for the detection of instability factors (e.g. transposons, mycoviruses), (5) test new application systems for effective targeting of the pathogen, (6) study the impact of the pathogens on target and non-target insects, (7) conduct field trials to demonstrate/evaluate the efficacy of the fungal biological control agents, and (8) address some of the criteria for the registration of insect pathogenic fungi. The BIPESCO team kept to the schedule and achieved the goals. **Task 1:** The BIPESCO team established appropriate test methods for conducting assays against target and non-target pests and methods for rearing these organisms. **Task 2:** Valuable data on morphological and physiological characteristics of the *Beauveria* spp. and *Metarhizium anisopliae* strains are now available. For example, there were qualitative and quantitative differences in enzyme and metabolite profiles of each tested *M. anisopliae* strain. Most of these enzymes were required for general metabolism (i.e. "house-keeping"), a few were shown to be important pathogenicity determinants such as the subtilisin, Pr1. This enzyme was a virulence determinant that played a major role in the infection process (i.e. cuticle degradation). Several Pr1 genes were identified and sequenced. No obvious link was established between production of the secondary metabolites destruxin and oosporein by *Metarhizium* and *Beauveria*, respectively. Conidia of attenuated cultures of *M. anisopliae* differed in their adhesion properties compared with those of the original, virulent strain. Conidia could increase or decrease in adhesive properties. Spontaneous mutants of *Metarhizium* were discovered in which the Pr1 gene was silent (not expressed) or absent. Silencing or loss of the Pr1 gene only partly explained why entomogenous fungi like *Metarhizium anisopliae* become attenuated. Molecular and biochemical methods were developed to monitor Pr1 genes and enzymes, respectively. These methods have considerable potential for quality control (i.e. identifying *Pr1-deficient* or *Pr1-silent* mutants). Quantitative and qualitative differences in the carbon-nitrogen content of culture media influenced the growth, sporulation and virulence of *Metarhizium anisopliae*. Strains grown on carbon-rich media were usually less virulent than those grown in nitrogen-rich (but carbon poor) media. Increasing the osmolarity of the culture media usually increased/stabilised virulence but reduced conidial yield. The conidia were paler and less hydrophobic than conidia produced on normal media or on mycosed cadavers. Cultural conditions stabilising virulence of *Beauveria brongniartii* did not stabilise *Metarhizium anisopliae* demonstrating generic physiological differences between these fungal species. The BIOLOGTM microtitre plate system was used to elucidate the link between nutrition and virulence. Metabolism of specific carbohydrates and amino acids was species-related and altered depending on the degree of attenuation. Inter- and intraspecific variation in the carbon utilisation profiles was recorded. Some of the favoured

substrates included Monosaccharides and polymers (Tween 40 and Tween 80), disaccharides (e.g. sucrose) and sugar alcohols (e.g. xylitol). Extensive testing of liquid media revealed the Catroux-medium as the most suitable for mass production of *B. brongniartii*. Standard C- and N-sources could successfully be replaced by cheap compounds like molasses or brewer's yeast. **Task 3 and 4:** Knowledge on the production of aerial conidia, submerged spores and biomass of *B. brongniartii* facilitates extensive field trials but also extension of this knowledge to other mycopesticides. With June 2000 *Beauveria brongniartii*, produced on sterilised barley kernels, became registered as a biocontrol agent by the Austrian plant protectant legislation. The product name is Melocont[®]-Pilzgerste and is the first effective propagule against *Melolontha* spp. in an EEC-member state.

Task 5: Molecular methods and tools were established for characterising promising fungal strains. Protocols were developed for the molecular characterisation of fungal BCAs based on RFLPs of mtDNA, protease-gene differences and various amplified rDNA regions (18S, ITS-5.8S-ITS, 28S). These protocols were refined to optimize detection of *Beauveria* and *Metarhizium* at the species and strain levels and are now available for further scheduled demonstration projects. **Task 6:** The research work (field studies and laboratory work) could only be finalised with the assistance of the BIPESCO consortium from five European countries. One of the priorities in this task was to evaluate the susceptibility and behaviour of a range of non-target invertebrates to *B. brongniartii* and *M. anisopliae* strains. No side effects of the application of *B. brongniartii* were estimated on earthworms and collembolans in field trials and in bioassays.

Field trials were carried out in Austria, Denmark, Italy, Germany and Switzerland with different climatic and soil conditions. In Switzerland 40 kg ha⁻¹ of the commercial product of *B. brongniartii* were applied and the development of the fungus in the soil was studied using the isolation technique on selective medium and the *Galleria* bait method (GBM). The results were analysed in relation to presence/absence of the host and in relation to chemical and biological soil properties. The reduction of fungus density after two seasons post-application in absence of hosts was high and differed significantly from that in presence of host. A multiple regression analysis revealed a strong correlation of fungal growth with temperature, clay content and catalase activity. *Beauveria brongniartii* and *Metarhizium anisopliae* were re-isolated from soils after treatment with *B. brongniartii*. The results revealed a negative correlation between the two fungi. However, this interaction is believed to be an effect of the isolation methods and not the result of a competition in the soil.

Melocont[®]-Pilzgerste, the commercial product based on barley kernels colonised by *Beauveria brongniartii*, was tested against the common cockchafer *Melolontha melolontha* in large field trials over a period of 3–6 years. The barley kernel product was applied in pastures in Austria, Italy and Switzerland with a slit seeder at various times of the year. Highest efficacy of the product was achieved by incorporating the inoculum into the soil at a depth of 3 to 10 cm. The results of field trials in Austria conducted between 1995 and 2000 with the barley kernel product indicated that the density of *B. brongniartii* increased continuously after each of the five applications performed between autumn 1994 and autumn 1997. Microsatellite marker analysis allowed to demonstrate that the applied strain and re-isolated strains were identical. The application of the *B. brongniartii* barley kernel product resulted in a sufficient suppression of cockchafer populations after only 2 years of application. Similar results can be expected for the control of weevils with *Metarhizium anisopliae*.

The non-confidential information is already published and/or in press in 25 international, refereed scientific papers. Additionally, 109 BIPESCO contributions also provide information that will help end users (e.g. policy makers, registration authorities, industry) and the public in making more informed decisions regarding the use and the risks, if any, that fungal BCAs may pose to plant, human and animal health.

The BIPESCO consortium strengthened its activities to be able to draft two dossiers pertaining to the entomopathogenic anamorphic fungi *Beauveria brongniartii* and *Metarhizium anisopliae*, respectively. The dossiers contain information to the fungi intended for the use as a microbial pest control agent (BCA) against *Melolontha melolontha*, *Melolontha hippocastani*, *Phyllopertha horticola*, *Amphimallon solstitialis*, *Strophosoma* spp. and *Otiorhynchus* spp. The information is prepared lay-out conform to EU Commission Directive 91/414 Appendix IIB and IIB. Comments are included to show expert authorities which of the proposed methodology cannot/or partly be used for biological material. Additionally, selected BIPESCO teams intended to proceed their field studies in two EU funded demonstration projects. Additionally, the consortium was strongly committed to making the BIPESCO project a success and drafted two demonstration projects as a strategy to exploit the technology. The proposals will be submitted in the thematic priority area “network of excellence” defined in the sixth framework programme of the European Union.

A further EU RTD-project (QoK1-CT2001-01391), initiated in autumn 2001, goes back on the initiative of BIPESCO teams. The significant interest in developing fungal BCAs, and the limited information on risk-assessment of natural agents led to this novel proposal.

The BIPESCO consortium (co-) organised International Symposia as follows: (i) the Melolontha-Tagung 2000, 23rd Feb., 2000, Auer, Italy. (ii) “Bioactive Fungal Metabolites – Impact and Exploitation”; held 22nd–27th April, 2001 at the University of Wales, Swansea; and the Closing Meeting of BIPESCO FAIR6 CT-98-4105" , 24th January 2002, University of Vienna, Austria. Furthermore, the BIPESCO team was helping to the "Third Meeting of the *Melolontha* Subgroup IOBC wprs Working Group "Integrated Control of Soil Pests" which is to be held 24th-26th September 2001 in Aosta, Italy. Additionally, BIPESCO run an own homepage to inform the public/users about the research and control activities (<http://bipesco.uibk.ac.at>).

Summarising, this BIPESCO RTD project (FAIR6-CT98-4105) drew upon the proven skills of European scientists, including industrial partners. Their expertise and complementary skills ensured the success of this project. There was no doubt that this project gave value for money because the consortium was only requesting 64% of the total cost of the project (2.443.297 €). The consortium is convinced to continue their work in demonstration trials in the future which will help validate the efficacy and safety of these fungi for the control of chafers and weevil pests.