ASPECTS OF THE BIOLOGY OF ENTOMOGENOUS FUNGI AND THEIR ASSOCIATIONS WITH ARTHROPODS

By

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ABSTRACT

I investigated several aspects of the biology of entomogenous fungi (mostly Clavicipitaceae with few species of Hypocreaceae). My primary motive in this research was to gain an understanding of the interactions between entomogenous fungi and arthropods. My study included field collections and identification of entomogenous fungi from BC and a few collections from Perú and Idaho. I addressed some aspects of the interactions among arthropods and fungi, life histories of fungi under laboratory conditions, and observations of chemical changes of fungi growing in the presence of heavy metals.

About fifty entomogenous fungi were collected in the province, mainly as anamorphs, but this permitted isolation and cultivation of many species. Of special interest is a small group of fungi parasitic on spiders some of which may be new records for western Canada.

Interactions of entomogenous fungi and heavy metals yielded a cerebroside not detected, or known to be produced, in the absence of heavy metals. This compound showed antibiotic activity against *Staphylococcus aureus*. The induction of this cerebroside by exposure to copper also is a promising approach to obtaining new drugs, or to increase the yield of selected compounds, from these organisms. The biological activities of other extracts were assessed, demonstrating additional compounds of interest (e.g., antiviral, antibacterial, phototoxic and antifungal substances).

Cultures grown on substances rich in oils and proteins (nuts and seeds) appeared to induce development beyond the anamorph stage to early teleomorph form. No perithecia developed although large synnemata and relatively bright pigmentation were observed. The ability to induce complete development of ascocarps would be of laboratory interest in the possible production of substances from wild ascocarps. Cultures obtained in this study will be deposited in the Canadian National Culture Collection, Ottawa, in the Canadian Culture Collection (UBC).

Further research remains necessary to fully understand the relationship between teleomorph and anamorph stages of entomogenous fungi, their nutritional requirements, for the production of teleomorph stages under laboratory conditions, and particularly to establish systems that may allow a chemical exploration for new drugs.

The preliminary studies of anti-arthropod activity by entomogenous fungi were partially successful in controlling a variety of laboratory-reared and naturally growing arthropods. The production of bio-pesticides is currently of great interest because of the problems with chemical pesticides and environmental pollution.

Gaining a better understanding of the biology of these organisms will allow us to develop new genetic strains of species for both biopesticides and biosynthetic applications. Both kinds of substances can contribute to maintain the health and equilibrium of the coastal temperate rainforest of the Pacific Northwest.

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LIST OF ABBREVIATIONS

^{13}C-NMR	Nuclear magnetic resonance, carbon 13
^{1}H-^{13} COSY	Correlated spectroscopy, carbon 13
^{1}H-^{1}H COSY	Correlated spectroscopy, hydrogen
^{1}H-NMR	Nuclear magnetic resonance, hydrogen
AFLP	Amplified fragment length polymorphism
BWA	Butanol-water-acetic acid
CC	Column chromatography
CH_2Cl_2	Dichloromethane
$CHCl_3$	Chloroform
COSY	Correlated spectroscopy Y
CWD	Coarse woody debris
CZA	Czapeck media
DNA	Deoxyribonucleic acid
EF	Entomogenous fungi
EtOAc	Ethyl acetate
EtOH	Ethanol
FBS	Fast bombardments scan
HCL	Hydrochloric acid
HMBC	Heteronuclear multiple bond correlation
HPLC	High pressure liquid chromatography
HSV-I	Herpes simple virus, type one
MeCN	Acetonitrile
MeOH	Methanol
MH	Müller Hinton media
mm	Millimetre

MS	Mass spectrometry
MYP	Malt yeast peptone
NH_4OH	Ammonium hydroxide
PDA	Potato dextrose agar
PDA1	Photodiode array
PTLC	Preparative thin layer chromatography
R_f	Relative mobility
SAB	Sabouraud dextrose Agar
SEM	Scanning electron microscope
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
Tween-20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet light
V/V	Volume by volume
W/V	Weight by volume

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This thesis is dedicated to all my relatives and friends who lost their lives while I have been away from my homeland.

To all my relations.

CHAPTER ONE: General Introduction

Temperate rainforest ecosystems of the Pacific Northwest of North America have tremendous trophic structure and biomass. Before the end of the 19th century, these forests covered a multitude of environments and landforms of approximately 11.3 x 10^6 ha (Harris 1984) from northern California to Alaska. In British Columbia (BC), temperate rainforests support high species diversity from micro to macro organisms. The gradient of species richness and abundance is associated with complex patterns and processes on different temporal and spatial scales (Wiens 1989; Huston 1994). Historically, fire has been one of the main forces shaping these forests, but during the 20th century suppression of wildfires led to different changes in the landscape. Windstorms (Lynott and Cramer 1966), pathogens (Childs 1970), and anthropogenic activities (forest harvesting; Franklin and Forman 1987) have also played a major role in the changes and sometimes destruction of these forests.

Fungi have been systematically studied for the last 300 years. They have been associated with degradation and recycling of decaying organic matter, and the maintenance of healthy ecosystems. They are a source of food for vertebrates (e.g., humans, squirrels, deer) and invertebrates (e.g., mycetophilodes, slugs, snails). They are also sources of toxins in animal food and produce many secondary metabolites useful to humans. Of an estimated 1.5 million fungi (Hawksworth 1991), only about 80,000 have been described in the literature.

Entomogenous fungi (EF) are fungi that parasitize arthropods, specifically insects and spiders. The total number of EF species has been estimated to be around 15,000 or 1% of the total estimated number of fungal species today (Samson *et al.* 1988; Glare and Milner 1991; Hawksworth 1991; Roberts and Hajek 1992; Hajek and Saint Leger 1994). Groups with large numbers of EF are Hypocreales (520 sp), Entomophthorales (240 spp.), Septobasidiales (175 spp.), and Laboulbenieales (1730 spp.; Hawksworth *et al.* 1983; Weir and Hammond 1997). In the last 100 years, 4,000 scientific papers have been published on EF belonging to the Clavicipitaceae (Hywel-Jones 1997*d*). This is only a fraction of the total number of mycological

papers and only a few genera and species of EF have been selected for most experimental research (i.e., the Hypocreales and anamorphs thereof or of other groups that attack insects). Among the groups of fungi attacking arthropods are the Entomophtorales. Members of this group produce zygospores in sexual reproduction and their mycelia grow inside the hosts; the Septobasidiales and Laboulbenieales do not form hyphae within the hosts and they produce haustoria that penetrate in the host systems (digestive tract in Septobasidiales and circulatory system in Laboulbeniales). Among EF are *Cordyceps sinensis* (Berk.) Sacc, *C. militaris* (L. ex Fr.) Link.), *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Met.) Sorokin), *Hirsutella* sp., *Verticillium lecanii* (Zimm.) Viegas, *Paecilomyces variotii* Bainier, and *P. carneus* Duché & R. Heim. Most research has been conducted in laboratories, thus little is known about their ecology or biology.

Entomogenous fungi are parasites of arthropods. Canada's total invertebrate inventories report the presence of more than 155,000 species of insects and over 4,000 species of spiders (Pojar 1991). In BC alone, more than 35,000 species of insects and 600 species of spiders have been reported, with the most representative insect orders being Diptera (true flies), Coleoptera (beetles), and Hymenoptera (wasps, bees, ants; Pojar 1991). It seems reasonable to assume that this abundance of arthropods supports significant populations of EF. However, forest pathologists, entomologists, and mycologists have only occasionally collected EF in BC (Table 1.1). Among these collections are members of the genus *Cordyceps* (Fr.) Link and the anamorph genera *Paecilomyces* Bainer, *Beauveria* Vuill., *Verticillium* Nees and *Hirsutella* Pat.

The life histories of most EF probably include both anamorphic and teleomorphic stages. The *teleomorph* is the sexual morph or state of the fungus and is characterized by ascomata (Hennebert and Weresub 1977). It is often more demanding of appropriate conditions and nutrition for its development than the anamorph. The *anamorph* is the mitospore form of the fungus, characterized by the production of conidia (Hennebert and Weresub 1977) and/or sclerotia (Kendrick 1992). Although, the life histories of EF have not been elucidated in cultural studies, it is known that one or more types of anamorph spores can be produced by a species.

Haploid mycelia can convert the body of an insect host into a sclerotium-like mummy. This mummy may overwinter in many temperate species. Stalked stromata are characteristic of the teleomorph states of *Cordyceps* species, and these arise from the mummified host (or from a host ascocarp in the case of *C. capitata* Holmsk. (Ex Fr.) Link.).

Table 1.1 Number of strains, location, and substrate of species of entomogenous fungi collected in Canada. Isolates deposited at the National Culture Collection in Ottawa, Canada.

Species	Number of strains	Location	Substrate
Paecilomyces variotii	10	New Brunswick	*Picea* and *Acer*
		Ottawa	Pea Seeds
		Quebec	Lipstick
		Calgary	Radish seeds
		Ontario	House walls
		Alberta	Rapeseeds
		Alberta	*Pinus contorta*
		British Columbia	*Brassica* sp.
P. tunulpes	1	Quebec	Insect pupae
P. carneus	4	Quebec	Soil
		Alberta	Soil
		Alberta	Soil
		Alberta	Soil
P. farinosus	14	Ontario	Laboratory contaminant
		Baffin Island	Mites
		Alberta	Alpine soil
		Quebec	*Amanita virosa*
		Quebec	Spruce budworm
		Manitoba	Larval *Megachile rotunda*
		New Bruinswick	*Picea glauca* seeds
P. fumosoroseus	2	Quebec	Soil
		Ontario	Gypsy moth egg masses
P. inflatus	3	British Columbia	Garden soil
		British Columbia	Farm soil
		British Columbia	Vancouver Aquarium, Tropical Valley
P. lilacinus	6	Quebec	Soil
		Alberta	Soil
		Ontario	Winter wheat cv. Lennox
		British Columbia	Soil, Simon Fraser University
		Ontario	Laboratory contaminant
P. stratisporus	1	Quebec	Soil
Cordyceps hesteri	1	Ontario	Soil insect
C. ophioglosiodes	1	Quebec	Flies?
Beauveria bassiana	14	Alberta	Soil

The term *synamorph* applies to two or more anamorphs that have the same teleomorph (Gams 1982). The *holomorph* refers to the whole manifestation of the genotype including all its morphs and phases (Hennebert and Weresub 1977). The presence of teleomorphs and corresponding anamorphs existing at the same latitudes may only be substantiated in the field. Establishing the link between the sexual and asexual forms of EF remains problematic. Of more than 30,000 known Ascomycetes, only about 4,000 anamorphs have been matched to their respective teleomorphs.

Molecular fingerprinting has become a complementary tool to develop the molecular taxonomy of anamorphic fungi such as *Paecilomyces* (Tigano *et al.* 1995*a*; 1995*b*) and other EF. Figure 1.1 illustrates the taxonomic relationships of EF with other major groups of Ascomycetes.

Although the use of molecular tools has become helpful in determining the identity and taxonomic relationships of the anamorph stages of some EF at the genus level (Sugiyama 1994; Fukatso *et al.* 1997), these techniques have not been very successful in identifying individual species. For example, through the use of rDNA analysis it has been established that *Cordyceps sinensis* (Berk.) Sacc. is closely related to *Hirsutella sinensis* and clearly divergent from *Paecilomyces sinensis, Stachybotrys* sp., and *Tolypocladium* sp. (Chen *et al.* 2001; Liu *et al.* 2001). Additionally, *Metarhizium anisopliae* var. majus has been identified as the anamorph of *Cordyceps brittlebankisoides* (Liu *et al.* 2001).

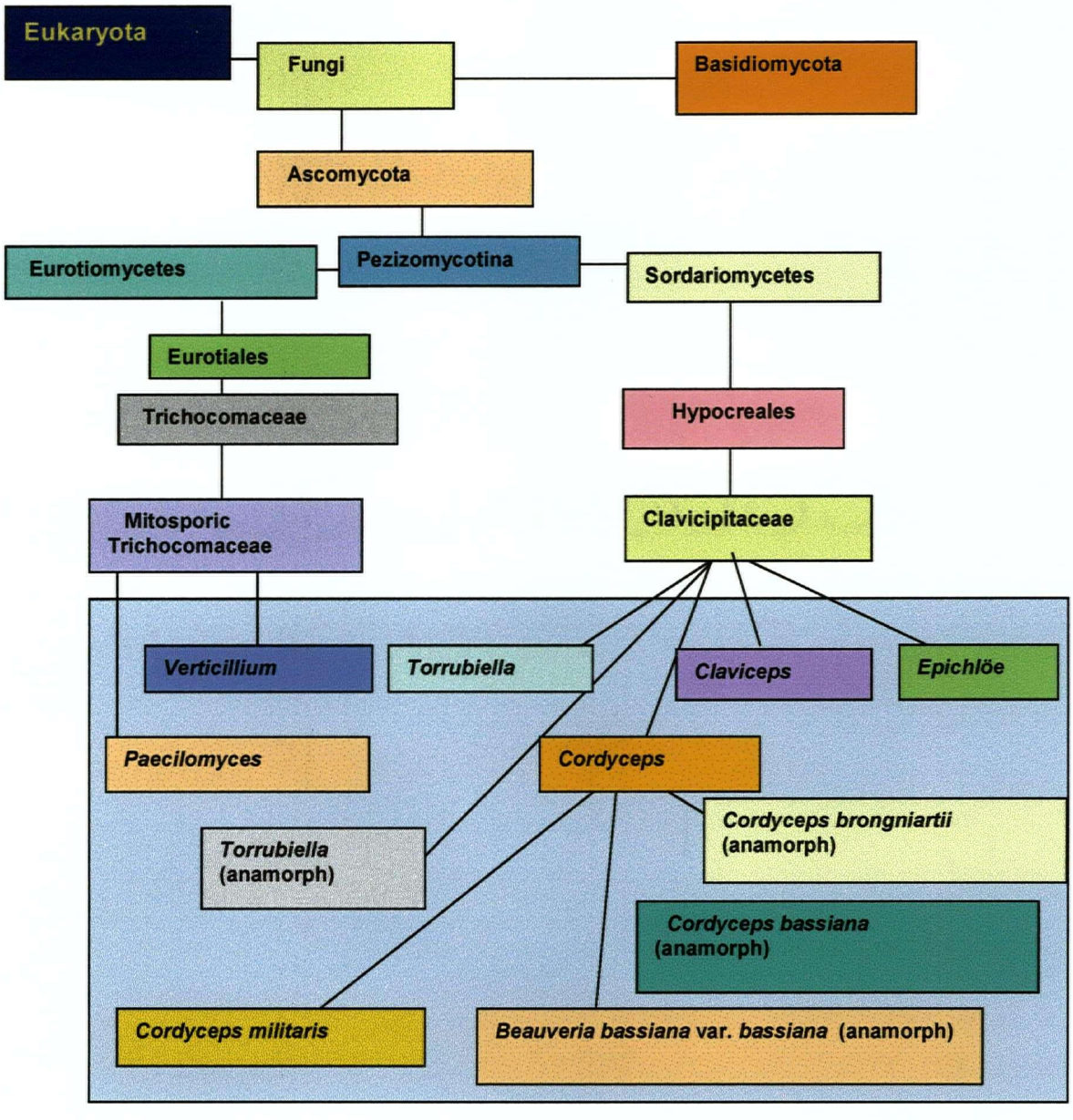


Figure 1.1. Taxonomic relationships of entomogenous fungi. Higher-level taxonomic relationships are shown for the groups as a whole, and some of the major genera and species of EF within the Clavicipitaceae. Anamorphs are included in the mitosporic Trichocomataceae. Diagram created with information from the National Centre for Biotechnology Information/US and the National Library of Medicine /National Institute of Health, 2001.

Fungi remain a vast untapped resource of novel metabolites. Numerous fungal metabolites have been reported as antibacterial or antifungal agents. Additionally, some of these metabolites may have antitumor, antiviral, or antiprotozoan activities. Today, the occurrence, distribution, and diversity of EF are directly affected by anthropogenic activities

such as agriculture, forestry, and industrial practices (Heliovaara and Vaisanen 1991; Puterka *et al.* 1994). The use of carbamide herbicides and increasing environmental pollution may negatively affect the occurrence, composition, and distribution of EF and other organisms.

Historical Significance of Entomogenous Fungi

Observations of diseased silkworms were made in China as early as 2700 BC; Aristotle mentioned diseased honeybees, circa 335 B.C. (Steinhaus 1956). In 1834, the Italian mycologist, Bassi discovered and associated the fungus *Beauveria bassiana* with the muscardin disease, a fungal infection of the silk worm, *Bombix mori* L. (Lepidoptera: Bombycidae). The name "*Cordyceps*" was first used by Link in 1833, to describe fungi growing on insects (Hawksworth *et al.* 1983). Fries and other mycologists referred to this fungus as *Isaria* Hill:Fr., a hyphomycete genus (Hawksworth *et al.* 1983). The term "*Cordyceps* and allies," was coined by Kobayasi in 1941, and is still used to describe members of the Clavicipitaceae growing mainly on insects (e.g., beetles, ants, butterflies, caterpillars) and spiders (e.g., Salticidae).

Mitchelli, Tillet, and Bassi's contributions to science were fundamental in the formulation of "the germ theory of disease," which remains a milestone in the biological sciences (Hawksworth *et* al., 1983; Kendrick 1992). Fifty years after the discovery of *B. bassiana*, Metchnikoff reported the interaction of *Metarhizium anisopliae* (Metch.) and the beetle *Anisoplia austriaca* (Hrbst.). Three decades later, Louis Pasteur's work in pathology began with a study of parasitized silkworms. Both Bassi and Pasteur suggested that microorganisms could be used in biological control (Stainhaus 1956). Other major contributions to our understanding of the fundamental biological processes of EF are those of Thaxter (1888), Petch (1923, 1931, 1932, 1937, 1944), Mains (1950, 1951, 1954), Evans and Samson (1982, 1984), Samson and Evans (1973, 1977, 1992), Kobayasi (1941), and Koyabasi and Shimizo (1963*a*, 1963*b*, 1976, 1981, 1982).

Cordyceps sinensis (Berk.) Sacc. and Traditional Chinese Medicine

Chinese medicine has a long history of use of EF. Most medicinal claims have been attributed to *Cordyceps sinensis*, the "cure-all-fungus." *C. sinensis* is a parasitic fungus that grows on larvae of lepidopterans (Kobayasi 1941). This well-known fungus has been used in Chinese traditional medicine for thousands of years (Zhu *et al.* 1998*a*; 1998*b*. *C. sinensis* is known as *tong tschong sha tso* which means, "worm in winter" referring to the fungus-infected insect, and "plant in summer" referring to the fungal fruiting body (Molitoris 1994).

In traditional Chinese medicine, many illnesses and medical conditions are treated with *C. sinensis,* including hepatic conditions, cardiovascular disorders, renal failure, immunological diseases, inflammatory conditions, and cancer (Jia-shi *et al.* 1998). As with many other traditional remedies, *C. sinensis* has been intensively studied. However, no unique active ingredient has been identified as being responsible for its medicinal effectiveness. The pharmacological properties of *C. sinensis* preparations have been suggested to be related primarily to bioactive polysaccharides, modified nucleotides, and cyclosporin-like compounds produced by this fungus.

In recent years in China, *Cordyceps sinensis* has been intensively collected from the wild (Yao *et al.* 2001). In addition, intentional fires, overgrazing, and shifting cultivation continue to destroy the alpine butterfly *Leptropteris* sp. whose larvae are the host for *C. sinensis*, ultimately threatening the existence of the fungus (Bhattarai and Croucher 1996). The new constitution of the Kingdom of Nepal 1991 recognizes the need to preserve the environment and to use natural resources wisely. At present, six non-timber forest products (NTFPs), which are recognized as threatened with over-exploitation, are banned from export in unprocessed medicinal products including *C. sinensis* (Bhattarai and Croucher 1996). However, the socio-economic and market demands may supercede the protection of wild *Cordyceps sinensis* populations. Cultivation of *C. sinensis* and other *Cordyceps* spp. has long been sought to reduce exploitation of natural populations (Liu *et al.* 2001) and to find new sources of natural products with biological activities (e.g., ergosterol peroxide, an antitumor agent from *C. sinensis* Bok *et al.* 1999). Therapeutic

preparations of EF from Chinese pharmacopoeiae have been introduced into western markets, but neither quality control nor regulations exist for the commercialization of most products.

Biologically Active Substances from the Hypocreales

Many species in the Hypocreales have unique interactions with a range of organisms including insects, spiders, grasses, and other fungi. *Claviceps purpurea* (Fr.) Tul (Clavicipitaceae) has a worldwide distribution, and several closely related species are parasitic on many temperate pasture grasses and cereals (e.g., rye, wheat, barley, and hybrids). In addition, members of the genus *Cordyceps* (Clavicipitaceae) have attracted the attention of western researchers in recent years, not because of their high chemical diversity but because unique bioactive compounds with pharmaceutical potential (i.e. immunosupressor and anti-cholesterol drugs) have been isolated from members of this genus. For example, Cyclosporine A (CysA) was discovered as an antifungal agent produced by the fungus *Tolypocladium inflatum* Gams. CysA is widely used as an immunosupressor agent in human organ transplantation. Unlike other immunosupressants, it has inhibitory effects primarily on the activation of T lymphocytes (Borel *et al.* 1977; Larsson 1980; Shevach 1985; Manger *et al.* 1986; Noble 1995). It is also effective in treating autoimmune disorders such as psoriasis and rheumatoid arthritis (Phillips 1991).

Tolypocladium inflatum was previously known *Trichoderma inflatum* (Dreyfuss *et al.* 1976) and as *Cylyndrocarpon luciderm* Booth (Borel 1986). *Tolypocladium inflatus* has been considered the anamorph of *Cordyceps subsessilis* (Hodge *et al.* 1996). Other members or relatives of these EF may be potential sources of immunosupressants and other drugs.

Potential of Biological Control and Mycopesticides

The demands to reduce of quantities of toxic substances released into the environment (i.e. pesticides, heavy metals, gases) have increased, and many areas of the world are experiencing the compounding effects of pollution and environmental and biological

degradation. Integrated pest management, in which natural enemies and pest arthropods interact, plays a significant role in plant and animal protection (Hoy and Herzog 1985). Different organisms have been used as agents of biological control, including bacteria, viruses, arthropods, and fungi.

Mycopesticides are fungi or fungal preparations (e.g., spore suspensions) used as biological agents to control and kill arthropods (Robert and Sweeney 1982). In general, mycopesticides have a lower kill rate than chemical insecticides, but this does not necessarily mean a reduction in crop protection (Jeffs *et al.* 1997). The reliance on chemical insecticides for control of mosquitoes and other insects led to a number of adverse environmental consequences (e.g., the use of DDT and its effect on wild and beneficial animals; Lawrence *et al.* 1994, 1995).

There was little research on biological control by microorganisms in the first half of the 20th century (Lacey and Goettel 1995). The discovery of the insecticidal properties of *Bacillus thurengensis* Berliner (Tanada and Kaya 1993) continues to have a major impact. The commercial development and use of EF for controlling arthropods has lagged far behind that of *Bacillus thurengensis.* Inoculation of test arthropods with EF has sometimes been successful. The most common and useful method of inoculation has been the use of spore suspensions (Lacey and Goettel 1995). Positive results from pest management programs require the survival of non- targeted insects and other organisms in contact with the control agent (e.g., strains of *P. farinosus* capable of killing the cockroach *Blattella germanica* L.; Zukowski and Bajan 2001).

Heavy Metals and Entomogenous Fungi

The increasing awareness and risks of accumulating heavy metals in the environment has led to a quest for new and improved "clean" technologies (Bakkaloglu *et al.* 1998). An understanding of how microorganisms tolerate heavy metals can provide insight into strategies

for their detoxification or removal from the environment (Smith 1975; Briuns *et al.* 2000; Bakas 2001; Gessel 2001).

Fungi are extremely efficient scavengers for mineral nutrients, and valuable as symbionts of trees and other plants. Fungi are sensitive to temperature, oxygen, carbon dioxide, pH and ammonia fluctuations in their environment. The availabilities of many minerals ions (e.g., copper and iron) are pH dependent; mechanisms for obtaining and sequestering such elements have evolved in many fungi. Any changes in normal biological pathways cause modification in the production of metabolites. Sterol synthesis seems to be altered by the presence of Cu^{++} in the media, leading to disturbances in the membrane function and K+ efflux (Tarhanen *et al.* 1996, 1998). The levels of ergosterol may depend on the metal concentration in the media, indicating that heavy metals play an important role in fungal decline (Seitz *et al.* 1979), which may be compounded by other environmental factors such as acid rain, use of pesticides (Urs 1967; Storey 1986), and other anthropogenic disturbances.

Objectives

In this thesis, I describe the results of an investigation into the biological activity of entomogenous fungi, the potential for their use in biological control, and their resistance to heavy metals. My primary motive in this research was to gain an understanding of the interactions between EF and arthropods, but some experiments dealt with biological activity of EF products against other organisms. The tolerance of EF to high concentration of metal ions departs from the main goals of the research.

My objectives in this research were to:

1) Survey BC entomogenous fungi in the field and establish a culture collection,
2) Cultivate *Cordyceps* species, including isolates obtained from culture collections, collected sporocarps and infected insects,
3) Inoculate experimental arthropods with EF to assess their potential role as agents of biological control,

4) Examine the antibiotic, antifungal, phototoxic, and antiviral activities of EF,
5) Isolate and characterize the main bioactive compounds, and
6) Evaluate the growth of EF under heavy metal conditions to explore their potential use in bioremediation.

Study Area

EF were collected from field locations within the coastal western hemlock (CWH) and sub-alpine mountain hemlock (SAMH) biogeoclimatic zones of south coastal BC temperate rainforest (Figure 1.2). These biogeoclimatic zones include wet maritime-montane (CWHvm2), mountain hemlock (MH), and moist maritime windward (Mhmm1) sub-zones (Meidinger and Pojar 1991). CWH occurs west of the coastal mountains, from sea level to 900m on inward slopes in the south and mid-coast (up to 1050 m on leeward slopes); mean annual precipitation is 2228 mm (1000-4400 mm); mean annual temperature 8° C (5.2-10.5° C; Meidinger and Pojar 1991). SAMH occurs above CWH, from 400 to 1000 m; annual precipitation ranges from 1700-5000 mm (20-70% is snow), mean annual temperature ranges from 0.0-5.0° C (Meidinger and Pojar 1991).

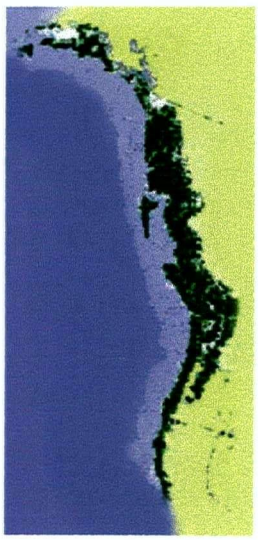


Figure 1.2. Collection areas for entomogenous fungi within the coastal western hemlock and sub-alpine mountain hemlock biogeoclimatic zones in British Columbia (shaded green).

EF were obtained in the field from the Capilano watershed, Cypress Provincial Park, the University of British Columbia Endowment Lands (Pacific Spirit Park), Ladner, the Squamish, Elaho, and Mamquam River Valleys (Coast Mountains; 49° N, 122°W; Figure 1.3), and the Morrell Nature Sanctuary (Nanaimo, Vancouver Island). Fourteen collections were obtained at sites outside BC as follows: Idaho, Priest Lake, (Figure 1.4a; cedar western hemlock forest) and Perú, Loreto, Yarapa River, (Figure 1.4b; tropical rainforest).

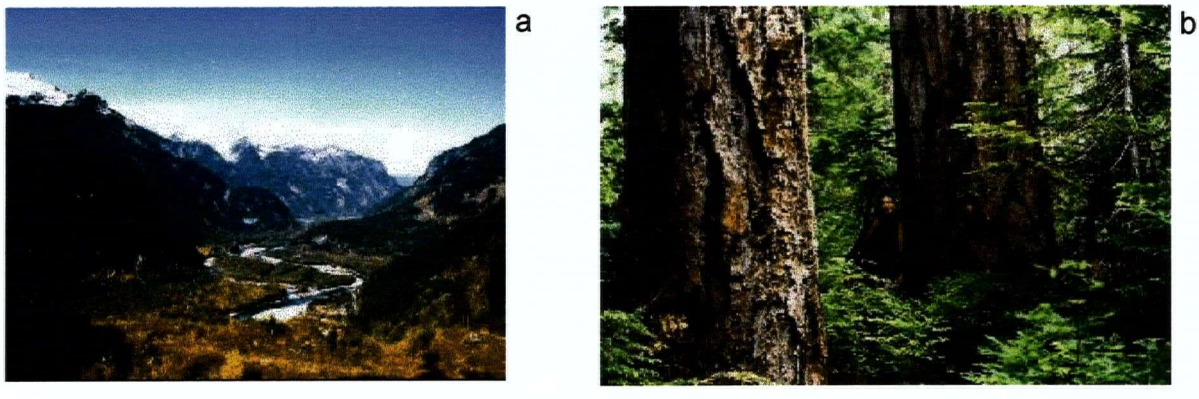


Figure 1.3. Coast Mountains, British Columbia. Study areas: a) Squamish River Valley, looking north towards Elaho River Valley, and b) Old-growth Douglas-fir forest in the upper Elaho Valley.

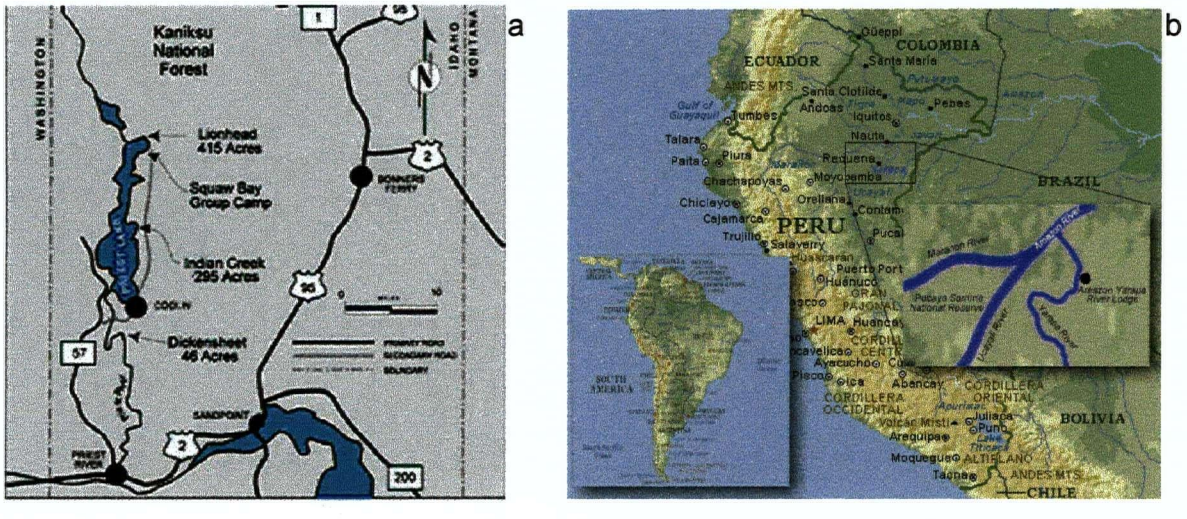


Figure 1.4. Locations of field collections areas for entomogenous fungi in a) Idaho and b) Amazonian Perú.

CHAPTER TWO: Collection, Isolation, and Cultivation of Entomogenous Fungi

INTRODUCTION

The diverse habitats present in the Coast Mountains of south coastal British Columbia (BC) have ecological conditions in which various groups of fungi, including entomogenous fungi (EF) can thrive. Some of the temperate rainforest fungi probably overwinter as conidia or mycelia in the soil, in other substrates such as decaying wood, or plant parts. Species of *Cordyceps* almost certainly survive as sclerotium-like hyphal masses in the host (mummies), the ascomata developing in the spring. For some small species, the development may occur in the spring, early summer, or fall but this is not known. It is known that some larger species produce sporocarps in the spring from buried mummies, but nothing is known about the survival of an individual conidium in the winter, and that may be rather poor. Nevertheless, hyphae survive freezing regularly in the laboratory (e.g., freezing in deep freezer, or freeze-drying are the two most common methods of maintaining cultures).

A limiting factor in the study of EF has been the lack of a clear understanding of culture requirements for fungi that grow on or within living insects and spiders. Knowledge of essential growth conditions is required to understand the nature of the fungus-host relationship. An understanding of the factors affecting inoculum persistence is needed (Omstad and Carruthers 1990) to predict the success of EF as potential biological control agents. Additionally, satisfying requirements for the production of teleomorphs in culture would permit genetic manipulation and selection to aid in the process of developing such agents.

Spiders, which feed on a wide variety of insects and other soft-bodied invertebrate animals, attack and subdue their prey using fangs to inject a poison. Most spiders are ecologically beneficial, while some are very toxic and may kill large mammals including humans. An understanding of the interactions between EF and spiders can be useful in the production of selective mycopesticides.

Some *Cordyceps* species and their allies produce useful compounds with medical, agricultural, nutritional, and industrial uses. Most are parasites of arthropods and their traditional medical uses are well known and described in the literature (e.g., *C. sinensis* used in traditional Chinese medicine; Jia-shi *et al* 1998). Some anamorphs of this group have been useful as agents of biological control (Burassa *et al.* 2001), and in the recovery of heavy metals (Bakkaloglu *et al.* 1998). Local strains of EF may be better adapted to local environmental pressures, and could be available for use as biological control agents in agriculture, forestry, and some industrial applications.

An improvement in culture techniques and an understanding of nutritional requirements of EF may lead to increased yield of natural compounds with specific biological activities (e.g., antibiotics). The number of existing EF living collections (or isolates existing in the Canadian National Fungal Collections, Central Experimental Farm, Ottawa) remains very limited.

Some filamentous fungi produce synnemata, structures composed of semi-compacted or strongly adherent groups of erect conidiophores bearing conidia on the apices, and in some cases, both on the apex and laterally. The production of synnemata *in vitro* may be a helpful taxonomic determinant for anamorphs of EF. Conidia borne on these clustered conidiophores making up the synnemata are probably the same as those on single conidiophores. The production of ascocarps *in vitro* offers many potential uses such as the production of inoculum, and breeding of appropriate strains for specific purposes. With the appropriate manipulation of environmental conditions and media nutrients, these synnemata should produce perithecia rather than the conidial state.

Claviceps Tul. and *Epichlöe* (Fr.) Tul. are two genera in the Clavicipitaceae. Species of *Claviceps* have a wide distribution and are considered parasites of grasses and sedges. Members of *Epichlöe* are ascomacetous fungi that appear to be symbionts of grasses. They are seed transmitted and considered monophyletic (Kaldu *et al.* 1997). *Epichlöe festucae* Leuchtm. Schardl & M.R. Siegel, which is a common symbiont of the grasses *Festuca, Lolium*, and *Koeleria* (Schardl 2001), produces the anti-insect alkaloids, peramine and loline, and the

anti-vertebrate alkaloids, lolitrem B and ergovaline. It also has an efficient vertical transmission via host seeds, a mildly pathogenic state associated with the *E. festucae* sexual cycle, and plays a role in improving the survival of host plants (Schardl 2001). *Acremonium* Link is a form genus, a genus created to include anamorphic species of different fungi, of which members are grass symbionts with unknown links to teleomorphs. It is generally considered a highly polyphyletic form genus containing distantly related fungi (Glen *et al.* 1996). Some species of *Acremonium* are probably anamorphs of ascomycetes belonging to the Clavicipitaceae (Glen *et al.* 1996).

In this chapter I describe collections from a survey of EF in BC, provide photographs of the representative groups of EF collected, and evaluate a number of substrata for possible teleomorph production. I also describe procedures for establishing a culture collection, and cultivation of *Cordyceps* spp. and EF anamorphs to examine the teleomorph stage in culture. Data obtained from free fatty acid analysis and rDNA sequencing of selected EF isolates were used to support morphological identification. This research contributes to enlarge the existing living culture collections of the Canadian National Fungal Collections in Ottawa.

OBJECTIVES

1. To survey BC entomogenous fungi in the field and establish a culture collection.

2. To cultivate *Cordyceps* spp. and EF anamorphs from field collections so that all life history stages, especially the teleomorph stage are formed in culture.

STUDY AREAS

Most collections were obtained from field locations in south coastal BC (Figure 2.1). Additional collections were obtained from sites in Idaho, U.S., and the Lower Amazon Basin, northeastern Perú. A detailed description of study areas can be found in Chapter One.

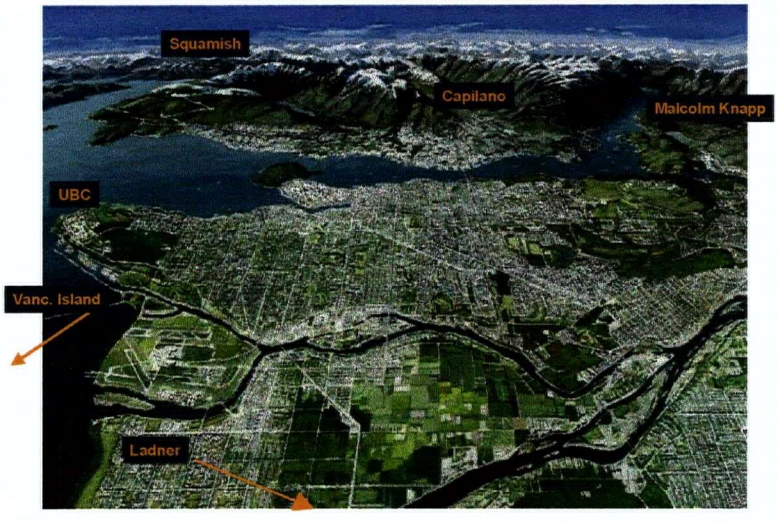


Figure 2.1. Entomogenous fungi collection sites in south coastal British Columbia.

METHODS

Field Collections

EF were collected in the field from 1998 to 2001. Monthly collecting trips were carried out from November to April, and weekly collections occurred during spring (March to June) and fall (August to October). The June, July and August collections were focused on ephemeral species appearing after rainy periods.

Field collections included sporocarps of *Cordyceps* spp. and arthropods with possible sporocarps formation, or superficial fungal growth suggesting EF anamorphs. Initially, sampling was done randomly using visual encounter surveys. Later, the searches were narrowed to specific habitats (i.e. decomposing logs). A knife or a hatchet was used to remove loose bark

from coarse woody debris for visual examination. Samples were examined in the field using a magnifying glass, then placed in 20-ml scintillation vials, or sterile bags.

Cultures were isolated from field collections using MYP medium containing tetracycline to eliminate bacterial growth. If conidia were present they were transferred directly from the sample to the agar plates. Young colonies were transferred from initial isolation plates to MYP (Bandoni 1971) plates without tetracycline. Stock cultures were maintained in MYP slant stored at 4°C. Other media used included MYP with the addition of copper, SAB, PDA, and water agar plates with the addition of tetracycline (see Appendix A for media ingredients and description).

Specimens were photographed using dissecting, compound, and electron scanning microscopes (SEM). Most photographs were taken using a digital camera. Other images were acquired using a 1200 DPI scanner, or using an automatic Minolta camera, model *3Xi,* using 400 ASA film.

Inoculated plates were kept upside down in an incubator at 26°C. Young colonies were immediately transferred to MYP agar containing copper sulphate. Finally, the clean culture was transferred to MYP agar without any metals. Slants were prepared for all stock cultures.

The EF strains obtained from the Osaka Institute for Fermentation, Japan were used as controls in my experiments. Isolates were identified by microscopic examination of the sporulating structures, morphology, pigmentation, and other morphological characteristics. The rDNA of seven fungal strains were extracted, sequenced, and partial sequences were compared to gene sequences in the Genebank. Similarities to other EF are shown in Appendix B.

Voucher Specimens

Voucher specimens were dried and placed individually in cardboard boxes; small specimens were glued into individual boxes. These specimens were deposited in the Herbarium of the Botany Department, UBC. Each specimen was accompanied by information indicating the collection date, hosts, location, and associated habitat. Final identification of some collections were not available for inclusion in this thesis. Collection numbers and

provisional names have been assigned to vouchers and living collections. In many cases, the host could not be identified because of extensive decomposition (e.g., when the mycelia had covered most of the host body), or only parts of the host were found and collected. Fungi were identified using light microscopy, and some images were obtained using a Scanning Electron Microscope (SEM). Field collections were photographed using dissecting and compound microscopes with a digital camera.

Maintenance of Fungal Isolates and Stock Cultures

Stocks of fungal strains were maintained on MYP, PDA, and SAB (Hawksworth *et al.* 1983) at 4°C on Petri dishes and in slant culture tubes. Small scintillation vials (20 ml) were used for the preparation of slant cultures. Each strain was stored in triplicate on MYP medium. Water stock cultures were also prepared using Ependorff tubes containing 0.5 ml water (distilled and autoclaved). A small amount of mycelium taken from the edge of an active growing colony was placed in an Ependorff tube, sealed with parafilm, and stored at 4°C. The water culture stock method was developed mainly for basidiomycetes growing on wood, however it works well for a number of filamentous fungi.

MYP slants were inoculated with selected fungal strains and incubated at 26°C. The caps were tightened once growth was visible, and slants were then stored at 4°C. MYP plates were inoculated and incubated at 26°C. Because the incubation time varied between isolates, plates were checked daily. Established colonies were removed from the incubator and transferred to a 4°C refrigerator. Cultures to be used for chemical extractions were grown at room temperature (25°C $\pm$ 4°C) unless otherwise indicated.

The most useful media for the cultivation of EF were malt yeast peptone (MYP), malt agar (MA), potato dextrose agar (PDA), Sabouraud dextrose agar (SAB), Czapek (Dox) Agar (CZA), and water agar (WA) (see Appendix A).

Attempts to Produce Ascomata in Culture

To attempt the production of synnemata *in vitro*, I used field isolates from southern BC: *Paecilomyces marquandii* (Massee) S. Hughes, *Paecilomyces tunuipes* (Peck) Samson, *Verticillium* sp., and *Cordyceps militaris* strain 5711, obtained from the Osaka Institute for fermentation. I used different substrates including arthropods, seeds, and nuts as sources of nutrients for EF.

Walnut, peanut, poppy, sesame, and flax seeds were placed in separate glass Petri dishes containing 5 drops of water each and sterilized by autoclaving at 121°C (Autoclave, Amsco 3021) for 30 min. Seeds and nuts were aseptically placed on MYP medium and inoculated with selected strains of fungi. Plates were kept at room temperature (25°C ± 4°C) under diffuse fluorescent light and reflected daylight.

Entomogenous Fungi Inoculated on Experimental Arthropods

To determine whether teleomorphs might be produced on experimental arthropods as substrates, MYP agar plates were inoculated with selected fungi and incubated at room temperature (25°C ± 4°C). After two weeks, conidial suspensions were obtained from these plates by adding 50 ml of a solution of 5% Tween 80^{R} and gently rotating the plate. Conidial counts were made and the suspensions were adjusted to give a final concentration of $1x10^{6}$ conidia/ml. These suspensions were used to inoculate the experimental arthropods.

The inoculated pupae were placed in a deep Petri dish containing a bedding of sterile sphagnum moss or wet silica gel, or on water agar plates. Petri dishes were incubated at room temperature (25°C ± 4°C), under fluorescent light and diffuse sunlight. Other bedding materials used included sterile Whatman paper No.1, moist silica gel, or sawdust. These materials were autoclaved at 121°C for 30 min. EF strains obtained from the culture collection of the Osaka Institute for Fermentation, Japan, were also assessed using the above protocol.

Arthropod Maintenance

Laboratory-reared arthropods used in this study included *Spodoptera litoralis*, *Tenebrion molitor*, *Pseudoletta unipuncta*, *Christoneura rosaceana*, and *Trichoplusia ni* (Table 2.1). These arthropods are commonly used as test organisms when looking for natural compounds or testing chemicals that can be used to control agricultural and forest pests (e.g., *Trichoplusia ni*). Isolates were used to inoculate arthropods of different developmental stages to determine whether ascomata production varied with developmental stage. Larval arthropods were kept at room temperature (25°C $\pm$ 4°C) in sterile plastic containers. Water was added to maintain high humidity; a plastic platform was used to keep arthropods separated from water.

Spodoptera larvae were fed a daily diet of premixed food, and the containers were cleaned daily. Grasshoppers and crickets were fed fresh lettuce and bran, and were maintained at 28°C, ambient humidity, and a light regime of 12:12 L:D. Beetles were fed bran and kept in wood shavings. *T. ni* larvae were fed broccoli leaf, and *Oncopeltus* were fed an artificial mixture. *Christoneura rosaceana* was fed fresh spinach leaves, and *Pseudotella* sp. was fed a sterile sugar solution.

Table 2.1. Species of laboratory-reared arthropods and wild arthropods, and their stages used in experiments.

Laboratory-reared arthropods	**Stage of development**
Spodoptera litoralis Fabricius (Lepidoptera)	larvae
Tenebrion molitor L. (Coleoptera)	adult and larval beetles
Oncopeltus fasciatus (Dallas)	adult
Trichoplusia ni (Hubner) (Lepidoptera)	larvae
Pseudotella unipuncta	adult
Christoneura rosaceana	larvae
Commercially-reared arthropods	
Acheta domestica	3rd instars
Tenebrion molitor	larvae
Field collected arthropods	
Ground spiders (Salticidae) *Pardosa* sp.	adult
Grasshoppers – *Melanoplus* sp.	adult

Free Fatty Acid Analysis of Selected Entomogenous Fungi

Plates containing MYP medium and a sterile cellophane membrane were used to grow the fungal mycelia separate from the solid medium. Selected isolates of EF were used in this experiment (See Table 2.1). The membranes were placed on the agar surface, an agar disk was cut from an active-growth area of a fungal colony, and mycelia were placed upside down in the middle of each plate. These plates were kept in the dark, in an incubator set at 27°C ± 2°C for a week. The cultures were allowed to grow for another week in the light, and at room temperature 25°C ± 2°C. After two weeks, a small amount of mycelium was harvested and extracted with 2 ml acidic alcohol (1N HCl MeOH). The sample was centrifuged for 10 min at 2000 xg, and 200 µl of hexane were added to the sample and allowed to separate into two layers. The fatty acids were contained in the hexane layer, which was collected and placed in a 5 ml glass vial, one ml of 10% NaCl in MeOH were added, and the vial was tightly capped and kept in an oven at 85°C for 90 min. This hexane fraction was used to prepare the samples for gas chromatography (GC). A two-dimensional TLC method was developed for initial visualization of the lipids.

RESULTS

Survey of Entomogenous Fungi in British Columbia

Fungi were often found on dead and mummified arthropods, under the bark of coarse wood debris, under logs, attached to leaves, and sticking out of leaf litter. Half of the collections came from logs different stages of decomposition (Table 2.2). Logs with soft and detachable bark were easily examined. Parasitized larvae and adult beetles were sometimes found covered with white mycelia. Some spiders were found attached to leaves. The areas of collection were selected based on accessibility and habitat. A large percentage of the fungi included in this study were isolated from specimens collected at the University of British Columbia. Several groups of EF are represented on this campus alone.

Table 2.2. Habitats where entomogenous fungi were collected, hosts on which fungi were obtained, number of collections, and percentage of total collection in British Columbia.

Fungi Isolated	Habitat	Host/Substrate	Number of Collections	Percent of Total Collection (n=53)
Paecilomyces	galls	wasp	2	3%
Paecilomyces, Torrubiella, Verticillium, Cordyceps	leaf litter	insect parts, spiders, caterpillars, beetle larvae, cocoons	11	20%
Torrubiella, Verticillium	logs	spiders, beetles, ants,	26	50%
Paecilomyces, Beauveria	branches	beetles	3	5%
Torrubiella, Gibellula, Cordyceps	leaves	spiders, insects	5	10%
Paecilomyces, Cordyceps, Beauveria	soil	insect parts	5	10%
Epichloë, Claviceps	grasses	grains, inflorescence	1	2%

Field Collections

My photographs of EF (Figures 2.2 – 2.18) represent field collections from 1998-2001. Two small collections are also included from Priest Lake, Idaho, U.S., and the Lower Amazon Basin, Yarapa River, northeastern Perú.

Figure 2.2. *Torrubiella arachnophila.* Voucher 96-21. Growing on a spider. Anamorph. Collected in the Squamish Valley, BC. June 8, 1998.

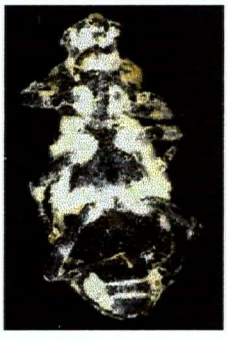


Figure 2.3. *Beauveria bassiana*. Voucher 84-2. Growing on a beetle. Anamorph. Collected in the Mamquam Watershed, Squamish area, BC. October 9, 1998.

Figure 2.4. *Gibellula pulchra.* Voucher 98-3. Growing on a spider. Teleomorph. Collected in Pacific Spirit Park, UBC, BC. July 17, 1999.

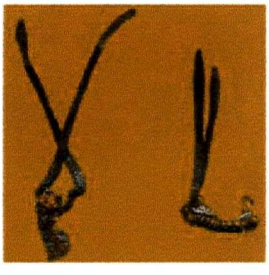


Figure 2.5. *Cordyceps nigrella.* Voucher 100-3. Growing on beetle larvae. Teleomorph. Collected in Tofino, Vancouver Island, BC. June 20, 2001.

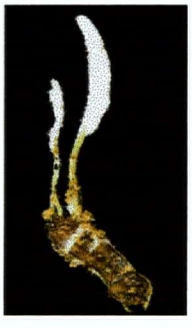


Figure 2.6. *Paecilomyces inflatus.* Voucher 98-2. Growing on beetle larva. Anamorph. Collected in Pacific Spirit Park, UBC, BC. July 17, 1999.

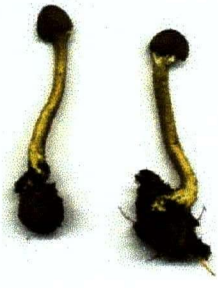


Figure 2.7. *Cordyceps capitata.* Voucher 82-25. Growing on *Elaphomyces granulatum*. Teleomorph. Collected in the Squamish Valley, BC. August 22, 1998.

Figure 2.8. *Cordyceps tuberculata.* Voucher 01-04. Growing on a butterfly. Teleomorph. Collected in the Lower Amazon Basin, along the Yarapa River, northeastern Perú. July 5, 2001.

Figure 2.9. *Torrubiella* sp. Voucher 01-02. Growing on a spider. Teleomorph. Collected in the Lower Amazon Basin, along the Yarapa River, northeastern Perú. July 7, 2001.

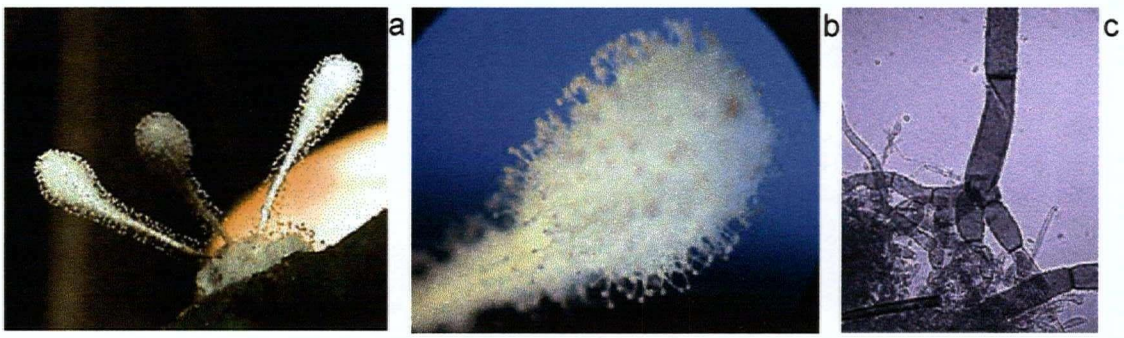
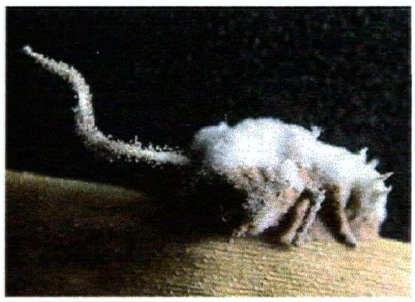


Figure 2.10. *Torrubiella mirabilis.* Voucher 01-08. a) Growing on a spider. Teleomorph. Collected in the Lower Amazon Basin, along the Yarapa River, northeastern Perú. July 7, 2001. b) aspergilloid terminal head, c) attachment to the host.

Figure 2.11. *Paecilomyces* sp. Voucher 94-1. Growing on a moth cocoon. Anamorph. Collected along Marine Drive, UBC campus, BC. October 11, 1998.


a

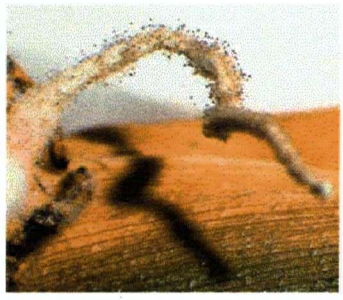
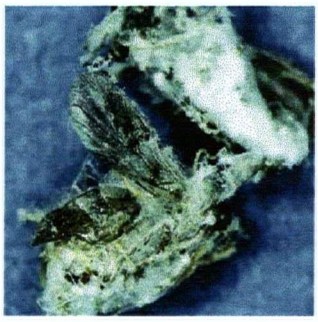

b

Figure 2.12. *Torrubiella raticaudata.* Voucher 01-09. a) Growing on a spider. Teleomorph. Collected in the Lower Amazon Basin, along the Yarapa River, northeastern Perú. July 3, 2001. b) Whip-like structure protruding from host.


a


b

Figure 2.13. *Paecilomyces* sp. Voucher 97-4. a) Growing on wasps. Anamorph. Collected in Ladner, BC. June 9, 1999. b) Rose gall with wasps.

Figure 2.14. *Gibellula* sp. Voucher 01-06. Growing on a spider. Anamorph. Collected in the Lower Amazon Basin, along the Yarapa River, northeastern Perú. July 7, 2001.

Figure 2.15. *Gibellula* sp. Voucher 98-3. Growing on a spider. Anamorph. Collected in Pacific Spirit Park, UBC, BC. August 29, 1999.

Figure 2.16. *Paecilomyces* sp. 85-14. Synnemata. Growing on a mummified caterpillar. Collected in the Capilano Watershed (GVRD), BC. October 10, 1998.

Figure 2.17. *Gibellula* sp. Voucher 80-15. Growing on a spider. Anamorph. Collected in Cypress Bowl, BC. June 19, 1998.

a

b

Figure 2.18. *Gibellula* sp. Voucher 100-1. a) Growing on a spider. Anamorph. Collected in Nanaimo, Vancouver Island, BC. October 22, 1998. b) Synnemata protruding from host, showing conidiophores.

Microscopic Documentation of Field Collections

I photographed the following images (Figures 2.19- 2.25) using a Zeiss bright field microscope and a digital camera. Colonies were grown on MYP medium and were one-week old when photographed. These represent some of the most common genera of EF collected during my study. All photographs were taken under oil immersion (100x). The most distinctive anamorph features are shown and listed in the legend of each figure.

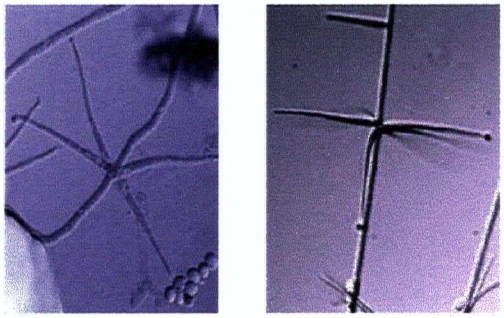


Figure 2.19. Whorled conidiophores in *Verticillium* sp.

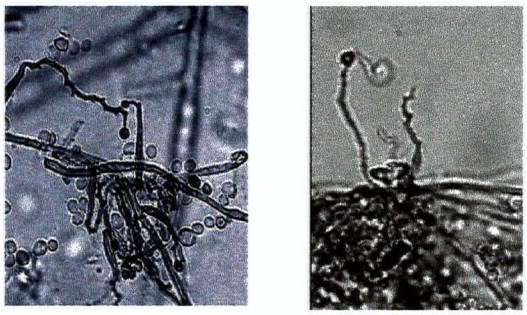


Figure 2.20. Sympodial conidiogenous cells arising from short swollen denticulated rachis of *Beauveria* sp. Teleomorph not observed or reported. Type species: *Beauveria bassiana* (Bals.) Vuill.

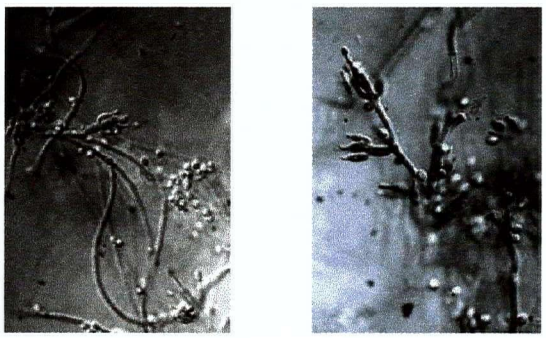


Figure 2.21. *Paecilomyces* sp. Penicillate with terminal whorls of phialides (cells with a swollen basal portion, tapering at end). Teleomorphs: *Byssochlamys* Westling, *Talaromyces* C.R. Benjamin, *Thermoascus* Miehe. Type species: *Paecilomyces variotii* Bainier.

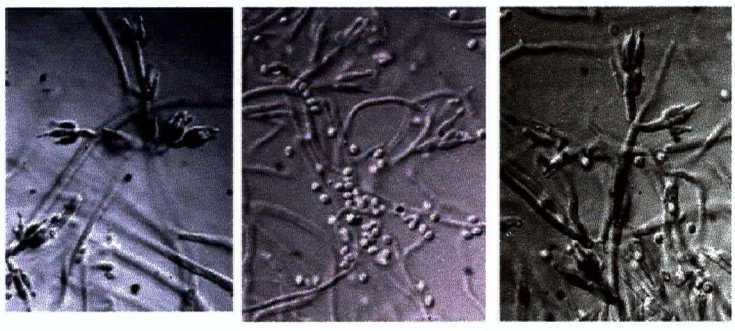


Figure 2.22. *Paecilomyces* sp. conideogenous cells bearing conidia.

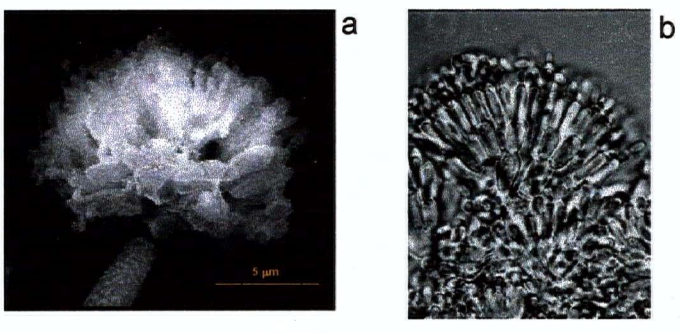


Figure 2.23. *Gibellula* sp. Compact terminal conidiophore. a) Electron Scanning Microscope, and b) oil immersion 100x.

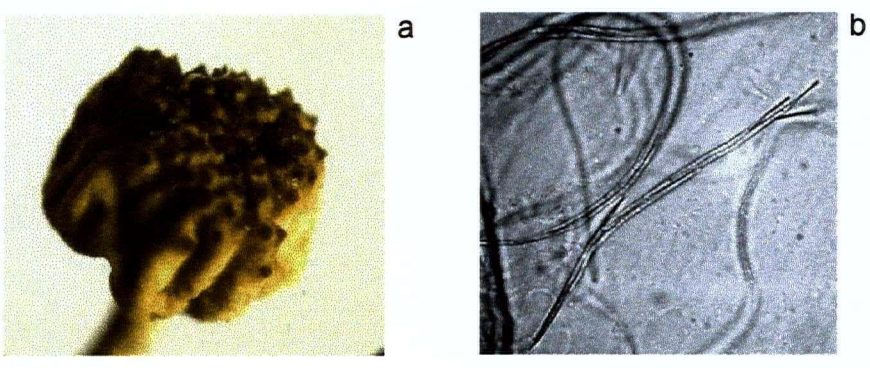


Figure 2.24. *Cordyceps loydii.* a) sporocarps, and b) fragmented spores. Oil immersion 100x.

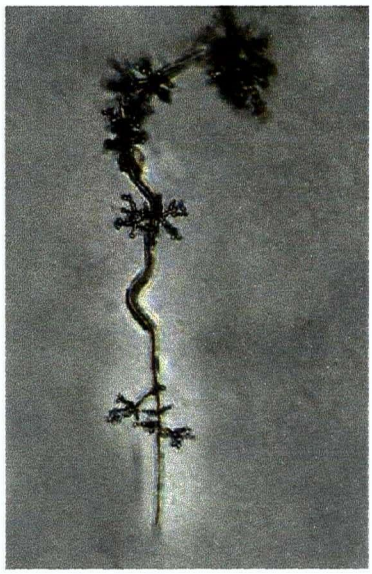


Figure 2.25. Anamorph isolated from *Cordyceps militaris* (01-07, Perú collection). Short phialides bearing spores.

Culture Collections

I obtained 22 isolates from EF collected in the field. These include *Paecilomyces*, *Beauveria, Verticillium*, and other anamorphs of *Cordyceps* and *Torrubiella* Boud. The isolates were tested for biological activities (see Chapter Three) and heavy metal tolerance (see Chapter Four). The following photographs (Figures 2.26 – 2.28) represent some isolates that were established from BC field collections. Cultures were two-weeks old when photographed.

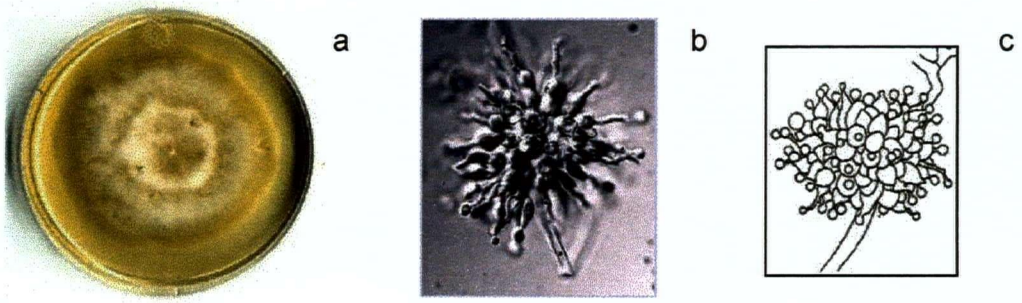


Figure 2.26. *Beauveria bassiana* a) colony growing on MYP medium, b) conidiophore showing phialides, and c) sketch of conidiophore and conidia.

Figure 2.27. *Paecilomyces inflatus* a) colony growing on MYP medium, b) conidiophore showing phialides, and c) sketch of conidiophore and conidia.

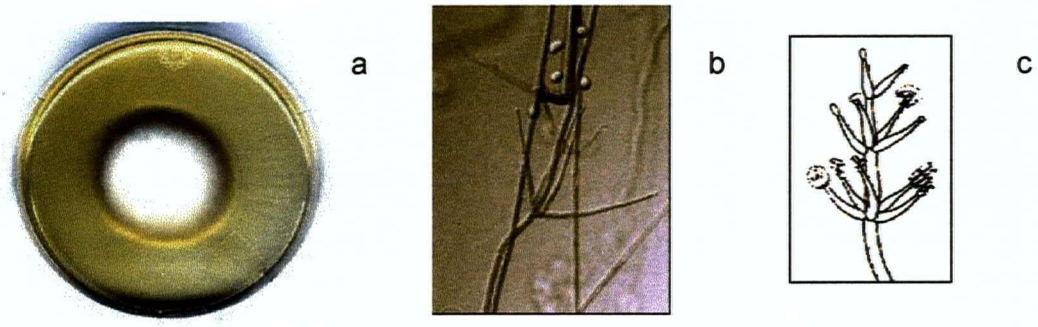


Figure 2.28. *Verticillium* sp. a) colony growing on MYP medium, b) conidiophore showing phialides, and c) sketch of conidiophore and conidia.

Attempts to Produce Ascomata using Seeds and Nuts

The inoculation of seeds and nuts did not produce ascomata, but different types of synnemata were produced depending on the media supplement and growing conditions. Synnematal production occurred using walnuts, pecans, and peanuts (Table 2.3). White synnemata grew from fluffy, cottony colonies growing on MYP plates with added walnuts; yellow-pigmented synnemata were more common when using pecans. Poppy and flax seeds were also assessed; tested EF grew mycelia but did not produce synnemata.

Table 2.3. Production of synnemata using different seeds and nuts. Inoculated plates were kept at room temperature (25°C ± 4°C), an equal number in the dark, and half exposed to light (Sylvania Grow-light), on a cycle of 12/12 hr L/D. X = synnemata; - = no synnemata, but small amounts of mycelial growth present; N/T = not tested. Synnemata developed under both treatments.

Fungal Strain	Walnuts	Peanuts	Pecans	Flax seeds
Paecilomyces marquandii (73-21)	X	X	X	-
Paecilomyces tunuipes (84-15)	X	X	-	-
Cordyceps japonica (9647)	-	-	-	-
Verticillium sp. (24-2b)	X	-	N/T	-
Cordyceps militaris (5298)	X	-	-	-
Paecilomyces sp. (85-16)	X	X	-	-
Paecilomyces sp. (80-14)	X	X	-	-

Most synnemata produced under these experimental conditions were cream colored; others were pale yellow, bright orange, and simple (Figure 2.29a), furcated (Figure 2.29b), or irregularly branched. They ranged in length from 1.0 to 3.0 cm. In other cases, pigmentation was enhanced (e.g., pecans)

a

b

Figure 2.29. *Paecilomyces* spp. synnemata produced on walnut mesocarp: a) single, no branching; b) furcated. Photographs under 10x dissecting microscope with Nikon 5x Coolpix digital camera.

Following the exhaustion of nutrients in the media, the morphology of synnemata varied in size and form as well as pigment production. An orange pigment was only produced when *Paecilomyces* sp. was exposed to nutrient-poor conditions (Figure 2.30). The age of the culture may also determine pigment production. The light regime used 12/12 hr L/D, enhanced the production of carotenoids. The control treatment, kept in dark conditions, produced less pigmentation and at a later age.

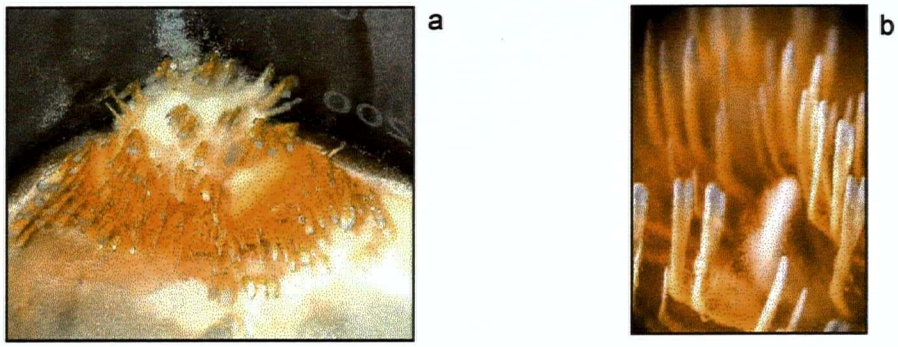


Figure 2.30. *Paecilomyces* sp. a) synnemata produced following a step down in nutrients, b) magnified to emphasize the intensity of the orange pigment. No ascocarps developed. MYP broth containing ¼ of nutrients.

Production of ascomata was attempted by using a number of substrates. Inoculated peanuts produced yellowish-white synnemata with a ropy shape (Figure 2.31). The strands were long and the pigmentation on the underside of the colony was bright yellow.

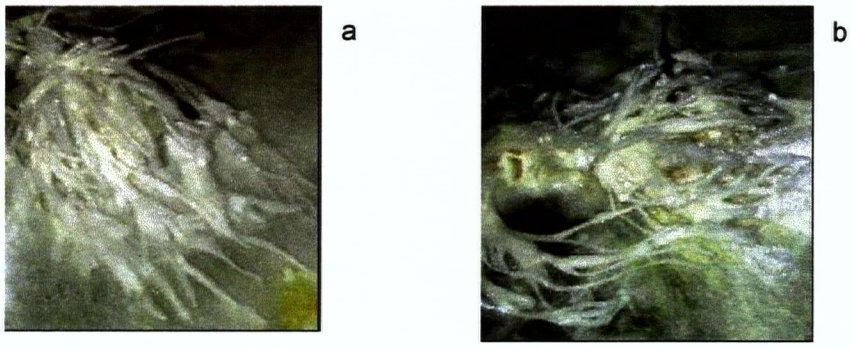


Figure 2.31. Synnemata of *Paecilomyces* sp. growing on peanuts. a) and b) illustrate two different strains of *Paecilomyces* sp. Colonies were six weeks old.

Synnemata also developed in liquid cultures, and different synnematal morphologies and aerial hyphae were observed. After two months, synnematal development in *Paecilomyces marquandii* was observed, but these structures stayed in a primordial stage for three months, when the experiment was stopped (Figure 2.32). The morphology of *Paecilomyces tunuipes* was affected by the shape of the container and by aeration (Figure 2.33). In both *P. marquandii* and *P. tunuipes*, no ascomata were produced, but synnemata developed above a thick, yellow mycelial mat.

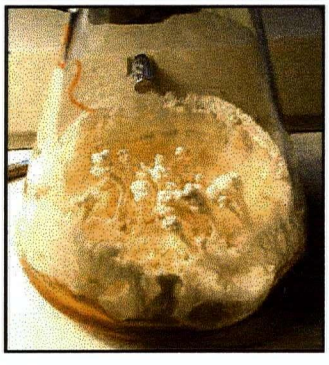


Figure 2.32. *Paecilomyces marquandii* was initially grown at room temperature (25°C ± 4°C) for one week, and then transferred to a cold room (4°C), under dark conditions. Culture was grown in MYP liquid medium.

Figure 2.33. A three-month-old culture of *Paecilomyces tenuipes* producing abundant aerial mycelia and synnemata. Culture was grown in MYP liquid medium.

Synnematal morphology, color, size, and development time varied depending on substrate, temperature, type of media (e.g., solid or liquid, MYP or PDA), and age of the culture. Best results were obtained with walnuts and pecans as substrate (Figure 2.34a). The pecans enhanced the production of pigmentation.

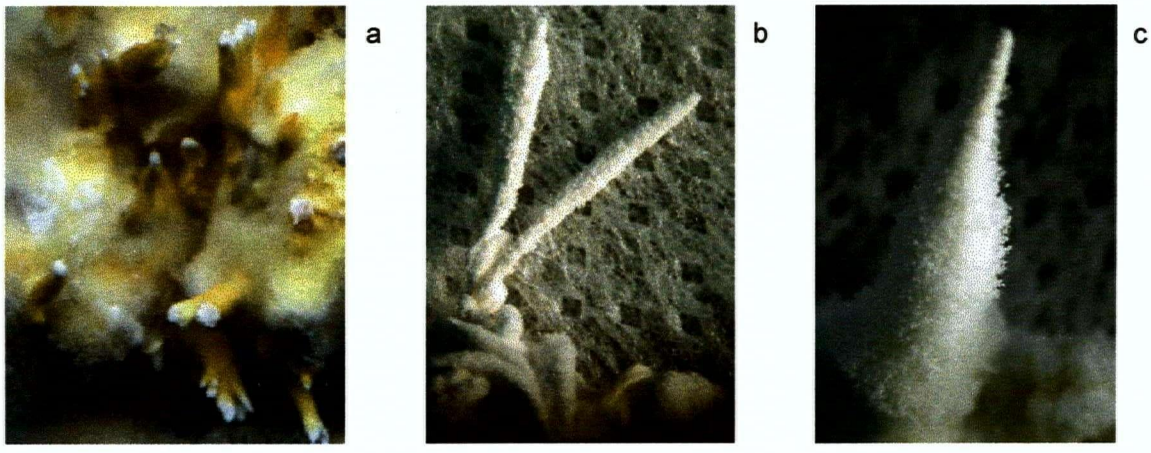


Figure 2.34. Synnemata of a) *Paecilomyces marquandii* (73-21) growing on pecans, b) *Cordyceps militaris* (5298) growing on walnuts, c) *P. marquandii* growing on walnuts.

With the exception of *Paecilomyces* spp, *Verticillium* sp., and *Cordyceps japonica,* no synnemata were produced on arthropods by any of the assessed strains. Four weeks after inoculation of the pupae of *Spodoptera litoralis*, synnemata began to appear. No perithecia formed. The moss bedding was replaced with silica gel, and a light regime of 16/8 hr L/D was introduced. Because silica gel is a hydrophilic material, relative humidity in the container dropped quickly, thus silica was not a good bedding material. Also, the grains of silica became attached to the fungal mycelia causing further dehydration. Best results were obtained by placing the pupae (inoculated host) in a deep petri dish containing water agar.

Arthropod Inoculation and Synnemata Production

Caterpillars of the moth *Spodoptera litoralis* (Figure 2.35) and the beetle *Tenebrion molitor* (Figures 2.38 and 2.40) were inoculated with different isolates from field collections obtained at the UBC campus. Members of the form genus *Paecilomyces* were the most aggressive in colonizing both wild and laboratory-reared arthropods. Arthropods were reared in the laboratory of Dr. Murray Isman, Agriculture, UBC. Other arthropods were tested and some were infected by the EF used in these experiments (see Table 2.4).

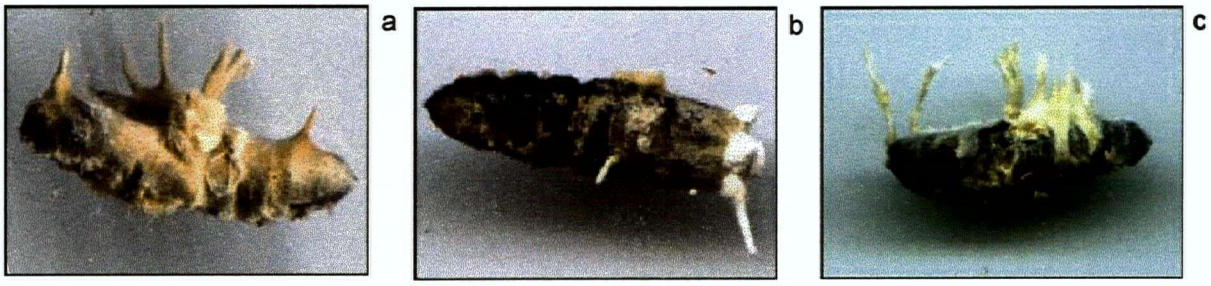


Figure 2.35. Synnemata growing on the cocoon of *Spodoptera litoralis.* Caterpillar was inoculated before metamorphosis occurred. The cocoons were kept in moist sterile chambers at room temperature (24°C) until synnemata developed. a) *Paecilomyces marquandii*, b) *Paecilomyces inflatus* (Burnside) J.W. Carmich., and c) *Paecilomyces tunuipes*.

Table 2.4. Synnemata production from arthropods inoculated with selected entomogenous fungi. X = synnemata produced.

Fungi	Host				
	Spodoptera litoralis	*Tenebrion molitor*	*Trichoplusia ni*	*Melanoplus* sp.	*T. molitor* (*larva*)
Cordyceps militaris					
C. japonica					
Beauveria bassiana				X	
Paecilomyces sp.	X	X		X	X
P. marquandii	X			X	X
Verticillium sp.					

Host mortality was observed in some of the inoculated hosts, but no visible mycelia were observed growing on the host. Percent survival of arthropods inoculated by fungi was examined and results are presented in Figures 2.36 and 2.37. *Paecilomyces marquandii* showed high percent mortality against *Christoneura rosaceana* and *Tenebrion molitor.* This fungus was originally isolated from a beetle larva.

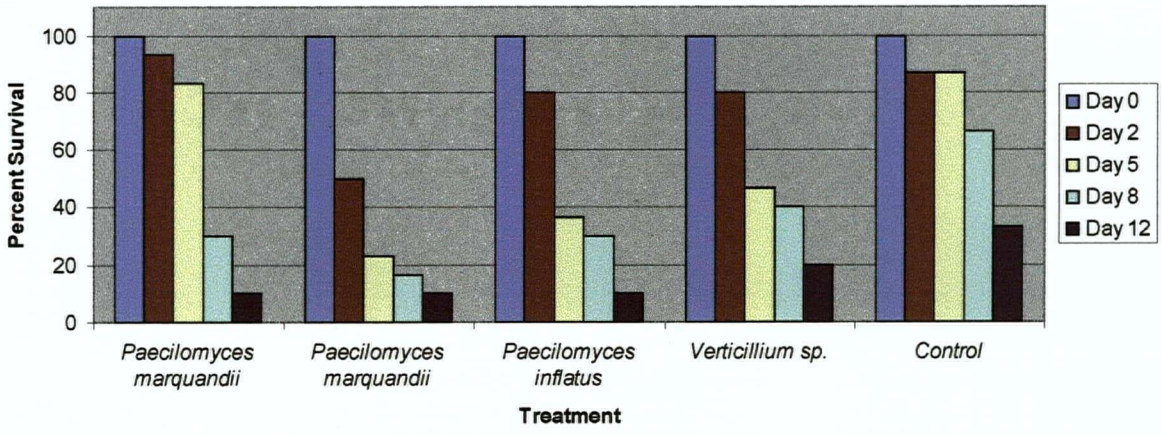


Figure 2.36. Percent survival of crickets, *Acheta domestica,* inoculated with four strains of entomogenous fungi collected in British Columbia. Total number of crickets on Day 0 was 30. The experiment duration was 10 days. Control is represented by pale blue line.

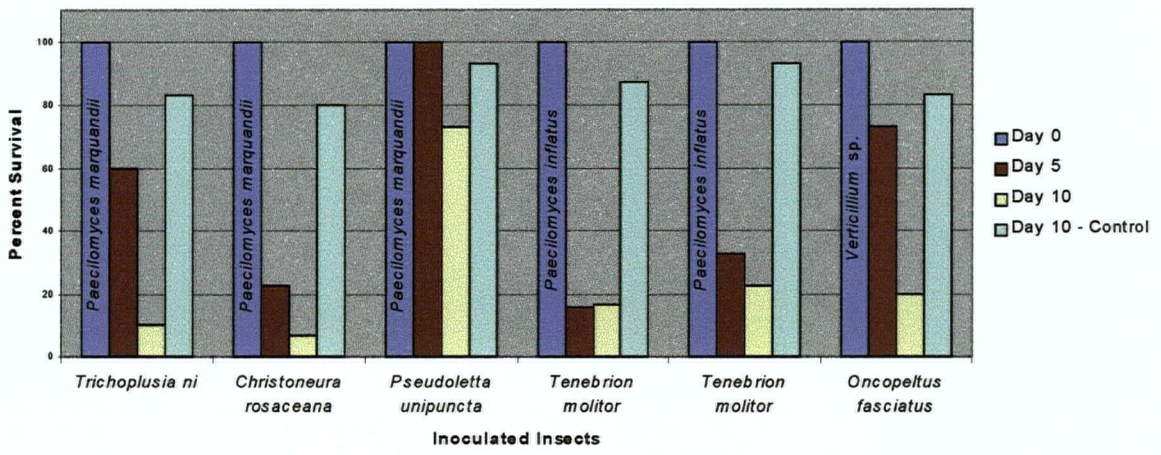


Figure 2.37. Percent survival of arthropods inoculated with entomogenous fungi. Fungi used to inoculate each arthropod species are identified in bars 'Day 0'. Total number of arthropods on Day 0 was 30. The experiment duration was 10 days. Control is represented by pale blue line.

Mycelia developed after 3-4 days and began colonizing most of the hosts. Five days after inoculation, a red secretion was produced from the posterior ends of the larvae. Similar secretions were produced by *Paecilomyces marquandii* growing on pecans (Figure 2.34a). The secretions were not collected for chemical analysis because of the potential risk of introducing contaminants into the experimental system, and to avoid disturbing fungal development.

Treatments were checked daily, and dead arthropods were removed and placed in sterile moisture chambers (kept at 28°C). The development of mycelia on the host was taken as an indication of death by mycosis. Percent mortality was assessed after 10 days.

During the first trial of this experiment, fungal contaminants (mainly *Trichoderma, Penicillium,* and *Aspergillus*) developed on the moss used as bedding material. This was probably caused by incomplete sterilization of the moss. Of all individuals in the *Spodoptera* control treatment, only two did not complete metamorphosis.

Among the isolates that colonized *S. litoralis* and *Tenebrion molitor* were *Verticillium* (strains 96-10, 98-2, and 100-1), *Paecilomyces marquandii* (73-21), *Paecilomyces tunuipes* (84-15a), and *Paecilomyces* sp. (80-14)

Three isolated strains from infested beetles collected at Priest Lake, Idaho, USA, were identified as *Beauveria bassiana.* These strains were used to inoculate adult beetles and larvae of *Tenebrion molitor,* one strain showed strong pathogenicity under experimental conditions.

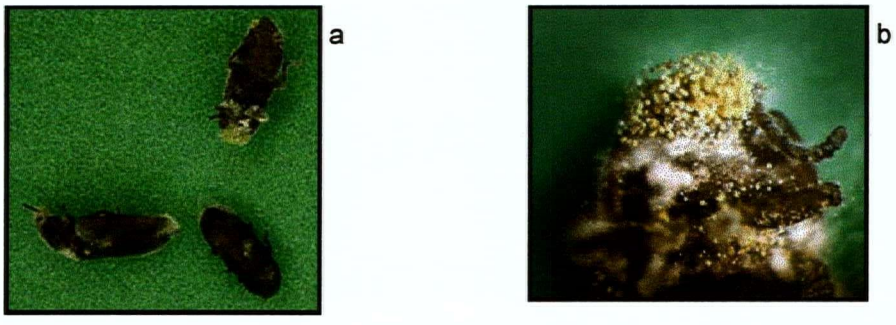


Figure 2.38. a) Adult beetles *Tenebrion molitor* parasitized by *Beauveria bassiana*. b) Fungal growth is more abundant at the joints and mouths of *T. molitor*. The isolate was obtained from an unidentified beetle collected at Priest Lake, Idaho, USA.

Larvae of the agricultural pest *Trichoplusia* ni were used to assess the pathogenicity of entomogenous fungus *Paecilomyces marquandii*. The spores of *Paecilomyces marquandii* germinated on the caterpillar and the mycelia colonized the whole body (Figure 2.39). No synnemata were produced. Some experiments could not be repeated because of the limited supply of arthropods.

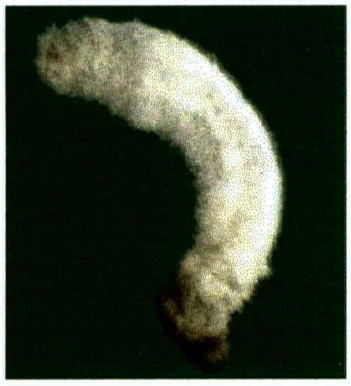


Figure 2.39. *Paecilomyces marquandii* (strain 85-15a) effectively colonized *Trichoplusia ni*, an agricultural pest.

Of the isolates of EF tested on beetles, only *Paecilomyces* sp. was capable of producing synnemata on the adult beetle, *Tenebrion molitor*. Synnemata were white and grew predominantly from the ventral surface of the beetle (Figure 2.40). Little mycelia were present over the rest of the body.

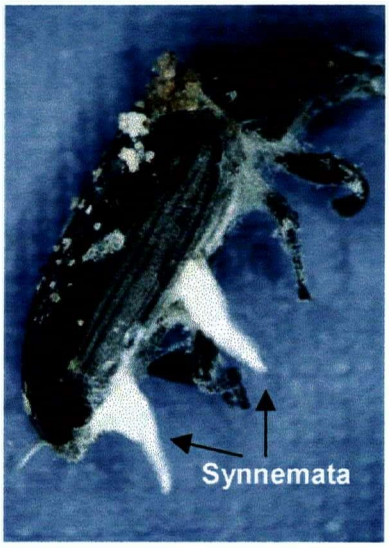


Figure 2.40. Synnemata developed from an adult beetle *Tenebrion mollitor* inoculated with spores of *Paecilomyces* sp. (95-10). The beetle was immobilized after three days of inoculation. Synnemata did not develop until two months after inoculation.

Figure 2.41. The artificial inoculation of *Paecilomyces inflatus* on carpenter ants did not result in production of synnemata, but mycelia grew on and inside the ant.

Free Fatty Acid Analysis

The most abundant fatty acids present in selected entomogenous fungal strains were 18:1 (9-octadecanoic acid; oleic acid), 18:2 (9,12- octadecanoic acid; linoleic acid), 16:0 (hexanodecanoic; palmitic acid), and 18:0 (octadecanoic acid; steric acid) in decreasing order (Table 2.5; see Appendix B, Figure B.2). The levels of 18:3 (19,12,15- octadecanoic acid; linolenic acid) relative to 18:2 are known to depend upon many factors, including culture mass.

Table 2.5. Free fatty acids present in selected entomogenous fungal strains. Columns A and B indicate two free fatty acids that were present but not identified.

Fungal Strains	Free Fatty Acids Present																	
	A	B		12:0	14:0	16:0	16:1 (9)	18:0	18:1 (9)	18:2 (9,12)	18:3 (9,12,15)	20:0	22:0	22:1 (11)	24:0	C	D	E
Beauvaria bassiana (BEET3)						x		x	x	x	x	x			x			
Cordyceps militaris (01-07)	x	x			x	x	x	x	x	x	x	x						x
Paecilomyces marquandii (73-21)	x					x	x	x	x	x								
Paecilomyces sp. (24-2B)	x	x				x		x	x	x	x	x				x	x	x
Paecilomyces sp. (85-14A)					x	x	x	x	x	x	x							
Paecilomyces sp. (95-2)						x												
Verticillium sp. (100-1)	x	x				x		x	x	x	x							
Standard				x	x	x	x	x	x	x	x	x	x	x	x			

In *Beauveria bassiana* (BEET3), 16:0 and 16:1 were found in lower concentrations than the average found for other fungi. Small amounts of 10:0 and 24:0 were also present in most tested strains. *Paecilomyces* sp. (24-2B) had the most diverse fatty acid composition, and most similar composition to that of *Cordyceps militaris* (01-07). *Verticillium* sp. (100-1) showed the highest amount of 18:0, and *B. bassiana* the lowest. No consistent differences in fatty acid composition among the strains tested were observed (See data in Appendix B). The small differences could not be used as special characters in species identification. However, these profiles may be useful for other purposes.

DISCUSSION

EF have not been studied intensively in most regions of the world, and the group remains essentially unknown from the standpoint of distribution, life histories, and genetics. However, available strains have been studied increasingly from the standpoint of chemistry because of the discovery of useful medical substances in the group. My research revealed a number of taxa of entomogenous fungi, including genera restricted to spiders and mites. The majority of EF in temperate rainforests collected in this study were anamorphic stages. Fewer teleomorph collections resulted from the surveys in BC. A short collection period in tropical rainforest yielded a larger proportion of teleomorphs than in temperate forests. Photoperiod and temperature may be partially responsible for the higher proportion of teleomorphic collections in the tropics.

The addition of media supplements such as walnuts, peanuts and poppy seeds may simulate the proteins and lipid content in arthropods. Walnut mesocarps contain between 63-70% oil, more than 90% of which is unsaturated fatty acids; the oleic acid content ranges from 12-20% (Savage *et al.* 2000). Also, phytosterol and vitamin E are part of the oil fraction, and may play a role in fungal sterol metabolism (Savage *et al.* 2000). I expected that these nutrients would contribute to the development of teleomorph stages in cultivation. Despite attempts, this

did not occur, but the experimentation was very limited and additional experiments involving temperature, light exposure, and variation in nutrient availability might produce successful results.

Fatty acids are precursors of vital structural and metabolic molecules. They are precursors of prostaglandins and related regulators. Fatty acid composition has been used as a key factor in genus or species identification, especially in bacteria, but also in *Aspergillus, Penicillium,* and *Mortierella*. I analyzed seven strains of EF for their fatty acid composition to determine if particular compositions are characteristic of a specific EF taxon. Free fatty acid composition examined by GC did not show dramatic differences among strains. Thus, free fatty acid composition analysis is not recommended as a taxonomic character among EF. However, rDNA sequencing support morphological identification of the selected strains (Appendix B).

The cultivation of *Cordyceps* has always been known to be problematic. Factors such as light regime, temperature, $C0_2$ and 0_2 levels, age of the culture and pH may have an effect on ascomata development, and general fungal growth and physiology.

The living collection resulting from this study could be used for further laboratory studies on EF as well as a taxonomic reference for future collections. The photographic documentation will complement existing information and databases on the fungi of the Pacific Northwest of North America. Some species remain to be fully identified.

Interesting EF identified during this research are those parasitic on spiders. Spiders parasitized by members of *Torrubiella* were collected in different locations in BC, including Vancouver Island, Cypress Bowl Park, and the Squamish Valley. These collections may be the first reports in BC, but this has not been officially confirmed. An additional new record for western Canada (though not parasitic on spiders) may be that of a species of *Cordyceps*, however final identification has not been completed.

Despite the amount of forest covering the landscape of BC (48.4 million hectares; Ministry of Forests Annual Report 2001), and the economic dependency on the forest industry (e.g., MOF Revenues 2000/01 = $1.3 billion) on these forests, arthropod-fungus interactions and their

biological role in maintaining balanced and healthy forests remains poorly understood in BC. The wealth of knowledge and expertise on arthropod biology is substantial, and existing collections in the province house a good representation of the local arthropods. However, entomogenous fungi remain underrepresented in herbaria.

It has become evident that EF play a significant role in the natural control of some arthropod pests (Tanada and Kaya 1993; Barson *et al.* 1994; Inyang *et al.* 1999). The discovery of *Beauveria bassiana* in 1836, by Agosto di Bassi lead to increased interest in EF. Since then, the search for entomogenous fungi for use in biological control has continued, but with limited success (Gillespie and Claydon 1989). Only a few mycoinsecticides have been developed based on EF spore suspensions and some of these are commercially available. Among these are Boverin (*Beauveria bassiana*), Mycar (*Hirsutella thompsonii),* Metaquino, Sorokin, Green muscle (*Metarhizium anisopliae*), and Vertalec (*Verticillium lecanii*; Kendrick 1992). *Beauveria bassiana* and *Metarhizium anisopliae* have shown positive results as mycoinsecticides, preparations of EF fungi used to kill insects or for tick control (Kaaya and Hassan 2000), and control of tsetse flies *Glossina* spp. (Kaaya and Munyiyi 1995). Monocerin and fusarentin 6,7-di-Me ether have been isolated from the entomogenous fungus *Fusarium larvaru*; both compounds have low molecular weights and have shown insecticidal activity against the blowfly, *Calliphora erythrocephala*. Destruxins A and B, dipsipeptides toxic to wax moth, *Galleria mellonella* Linnaeus, have been isolated from *Metarhizium anisopliae* (Roberts 1969). *B. bassiana* produces a variety of cuticle degrading enzymes including proteases, chitinases, and esterases (Gupta *et al.* 1991, 1992)

Worldwide, fourteen species of *Paecilomyces* are recognized as pathogens of various arthropods and nematodes found on plants and soil (Samsom 1974). High genetic variation has been reported among species of the form genus. For example, *Paecilomyces fumoroseus* and *P. liliacenus* show morphological similarities but are genetically and pathogenically different (Tigano-Milani *et al.* 1995*a*). Assessment of strain variation is still needed to select suitable strains for particular purposes (e.g., biological control, peptide production).

Little work was conducted on biological control until the first half of the 20th century with the discovery of *Bacillus thurengensis* (Tanada and Kaya 1993). The commercial development and use of EF for controlling of arthropods has lagged far behind that of *Bacillus thurengensis.* Because of the potential environmental benefits presented by the use of biological control agents, in contrast to the use of chemical pesticides, there has been much focus over the last 30 years on improving the survival of conidia (Smits *et al.* 1996) and large-scale production of inocula (Goettel 1984). The effect of myco and/or chemical pesticides on non-targeted microorganisms has been a concern for the last fifty years. The wide use of pesticides exerts a negative influence on non-targeted organisms such as soil fungi (Popraski and Majchrowicz 1995). Many chemical pesticides have shown toxicity and, in some cases, complete growth inhibition of EF (Urs *et al.* 1967; Horton 1980; Gardner and Storey 1985).

Local strains of EF may be good candidates for the development of biological agents to be used in pest management in BC forests. Because these fungi are already an integral part of the ecosystem they may reduce the potential negative effects on other organisms as shown by chemicals. Furthermore, EF in combination with other natural pesticides have resulted in an efficient biocontrol. The biopesticide Neemark (Azadirachtin) activity has been found to be compatible, *in vitro,* with the EF *Beauveria brongniartii* and to *Metarhizium anisopliae* (Vyas *et al.* 1992).

To investigate parasitism and pathogenic mechanisms, good experimental model systems are necessary where both parasite and host are easy to rear and handle. Numerous species of entomogenous fungi have been studied as potential biological control agents of arthropod pests (e.g., Butt and Wraight 1988; Butt and Humber 1989; Butt *et al.* 1996; Evans 1994; Jack and Jung 1998; Ibrahim *et al.* 1999). The selection of fungi for biological control requires a series of screening bioassays. The inoculation of wild locusts and spiders demonstrated that some entomogenous fungi have the potential to affect a wide range of arthropods. Because of the low sample size, my results do not allow me to make any strong conclusions. However, preliminary observations demonstrated that tested EF were not host specific, and caution should be taken

when using them as biological control agents. Ground spiders, Salticidae, were very susceptible to fungal infection by isolates of *Paecilomyces* spp. These experiments will need to be repeated to measure the degree of host specificity, mortality rate at different instars, and to assess levels of EF pathogenicity on wild populations of arthropods.

CHAPTER THREE: Screening of Entomogenous Fungi for Bioactivity

INTRODUCTION

The search for and isolation of biological compounds from fungi and bacteria has been carried out for many centuries. For a long time, the most common sources of fungal inocula were soil and plant debris. Thousands of biochemical studies focusing on the screening, isolation, and synthesis of fungal compounds have been produced (Gunde-Cinerman *et al.* 1993; Huang and Kaneko 1996; Hancock 1997,1998; Hancock and Chaple 1999). Many of these yielded compounds useful as foods, drugs, and in industrial applications. However, the intense uses of antibiotics have led to the development of organisms that are resistant to our present drug arsenal. The need for safe molecules to stop invasive infections of fungi, bacteria and viruses has increased over the years. The need for new sources of biologically active agents is widely acknowledged. EF are a group that remain poorly documented, although a wealth of fungal chemical diversity has been demonstrated and some bioactive compounds have been isolated. Some of these compounds have passed clinical trials and are currently used in treatments of particular diseases (e.g., Cyclosporin A, Cordycepin; Wasner and Pfleiderer 1995; Wasner *et al.* 1994, 1996, 1997).

Some currently available antifungal compounds act on targets on mammalian cells (Debono and Gordee 1994). However, some of these compounds may be cytotoxic and cause adverse drug interactions. This situation is similar for antiviral drugs, which need to be specific to the virus and not damage the host cell. Two general modes of action of known antiviral drugs are the prevention of viral penetration (e.g., amantadine) and the inhibition of DNA replication enzymes (e.g., acyclovir). Only a few compounds with antiviral activity have been isolated from fungi.

I screened extracts of EF for antiviral activity using the enveloped virus herpes simplex virus 1(Herpesviridae), a double stranded DNA virus in the herpes simplex group. This group includes chicken pox, shingles, and infectious mononucleosis viruses. The bacteria, fungi, and

virus used in this screening include organisms relevant to human diseases (e.g., *Staphylococcus aureus* methicillin resistant, *Escherichia coli*, and the yeast *Candida albicans*. These are also important model organisms used in many medical studies. The number of bioassays may not reflect the total potential of the tested EF, and other bioassays should be used in future studies.

OBJECTIVES

1. Examine the biological activity of EF, including antibiotic, antifungal, phototoxic, and antiviral activities.
2. Determine the amino acid composition in *Cordyceps sinensis*.
3. Screen for Cyclosporin A in EtoAc crude extracts of EF.

METHODS

Fungal Extract Preparation

Aliquots of 250 ml of MYP broth media were inoculated with selected EF and grown as previously described. The mycelia were extracted with 300 ml of hexane followed by three washes of 100 ml EtOAc over 24 hrs. The combined extracts were filtered and the volume reduced under pressure. The dried extracts were weighed and suspended in EtOAc:MeOH (2:1) to make a 10 µl/ml solution.

Antibacterial Bioassay

Antibiotic bioassays were performed by the disk diffusion method (Figure 3.1); Müller-Hinton (MH; Difco) medium was used to grow bacteria and Sabouraud dextrose (SAB; DIFCO) medium for fungi. Sterile paper disks (6 mm) were impregnated with 10 ml of the previously prepared crude fungal extract, and the solvent allowed to air dry in the fume hood.

An inoculum of each bacterial strain was suspended in a 10 ml glass tube containing 3 ml of Müller Hinton broth medium, and incubated overnight at 27°C, on a rotary shaker at 1200 rpm. Bacteria used in the bioassays included Gram (+) *Bacillus subtilis*, *Staphylococcus aureus* and Gram (-) *Pseudomonas aeruginosa*, *Escherichia coli*, *and Klebsiella pneumoniae*. The yeast *Candida albicans* was used to assess antifungal activity. Overnight cultures were diluted in MH nutrient broth to a cell density of 10^9/ml. The bacterial and fungal suspensions were used to inoculate MYP or SAB plates. Plates were prepared in duplicate.

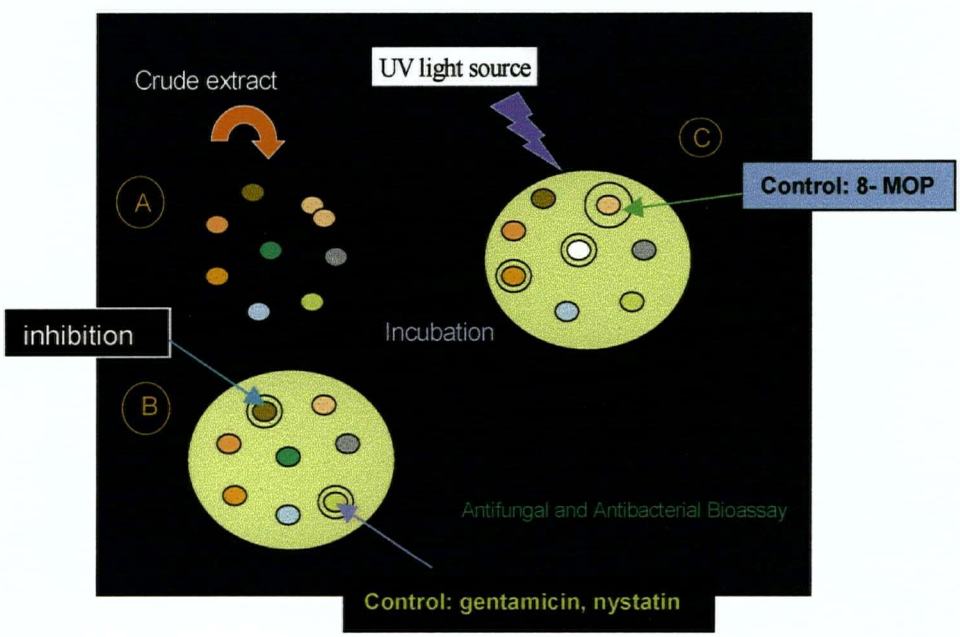


Figure 3.1. Diagrammatic sketch of Disk diffusion bioassay used to evaluate antibacterial, antifungal, and phototoxicity activities in EF. Extracts were used both with and without exposure to UV light to determine if photoactive antibiotic substances were present. A) An aliquot of each extract impregnated in a sterile paper disk and allow to dry; B) the disk was then placed on the agar surface of a plate inoculated with the appropriate test organism; and C) to assess phototoxicity a duplicate plate was prepared and exposed to UV light and incubated. Gentamicin was the control for antibacterial bioassays, nystatin was used as a control for antifungal bioassays, and 8-methoxypsoralen (MOP) was used for phototoxicity bioassays.

Photoactivated Antibiotics and Antifungal Substances

Prepared disks containing fungal extracts were placed on plates inoculated with selected bacteria and the yeast *Candida albicans*. One plate of each duplicate pair was exposed to long wave UV light for one hour at room temperature. Irradiation was provided by a set of four Sylvania F20T12-BLB lamps, which gave a measured incident energy of 5 W/m^2 and a maximum output of 350 nm. Duplicates of these plates were kept in the incubator without lights. After UV irradiation, all plates were incubated at 37°C and examined after 18 hrs. The diameter of the zones of inhibition around the paper disk were measured with a ruler and recorded. Samples showing zones of inhibition of microbial growth only after UV irradiation were recorded as phototoxic (+). Those samples with zones of inhibition in both light and dark were reported as antibiotic. A larger zone of inhibition in light-treated samples, compared to those maintained in the dark, was considered as enhanced activity (Wat 1980; Towers 1984, 1987).

Antiviral Bioassays

Crude EtOAc extracts of 18 fungal strains isolated from arthropods were assayed against the enveloped double stranded DNA Herpes Simplex Virus Type I (HSV-I). An African green monkey kidney cell line (Vero cells) was obtained from the American Type Culture Collection.

Vero cells were grown in Dulbecco's modified Medium (DEM) with 5% Fetal Bovine Serum (FBS; Gibco Life Science, Ontario) and 25 µg/ml gentamicin sulphate (Sigma), in 96-well microtest trays (Falcon) (Anani et *al.* 2000). An atmosphere of 5% CO_2 and 95% air at 37°C was provided using an incubator. Vero cells were considered ready for bioassays when a monolayer of cells was formed, about 5-7 days after inoculation.

Fungal extracts (EtOAc) were prepared from two-week-old MYP broth cultures. The medium and mycelia were separated by filtration, and both extracted three times with 100 ml of EtOAc, with a final hexane:acetone (80:20) wash. The fractions were pooled and dried under reduced pressure and dried extracts were stored at 4°C. For bioassays, the dried samples were suspended in MeOH, diluted to a concentration of 100 µg/ml, and passed through a 0.2 mm

pore diameter filter. The extract was further diluted 1:200 in DEM and dispensed in a 96 well plate containing a monolayer of Vero cells (Hudson et *al.* 1993, 1994).

The 96-well plates of Vero cells, treated with a serial dilution of the fungal extracts, were incubated at 37°C for 60 min, and then examined under the microscope for any cytotoxic effects caused by the extracts. Cytotoxic effects included changes in cell morphology and cell membrane damage (e.g., cytoplasm leakage). An aliquot of 100 µl of the virus suspension was added to each well and the plates were kept in an environmental chamber at 37°C for 30 min, with continuous shaking, and under long wave UV-A light (5 W/m^2, with an emission wavelength maximum of 350 nm, from four Sylvania F20T12-BLB lamps), or kept in the dark for 30 min. After these treatments, the plates were returned to the incubator at 37°C. Cultures were examined under a dissecting microscope after incubating for 72 hrs. The absence of viral cytopathic effects indicated 100% inhibition of the virus. Partial inhibition of the virus was considered a negative result. Positive results were recorded at concentrations showing minimum inhibitory effect. Acyclovir was used as a positive control against herpes simplex virus.

Screening for Cyclosporine A

A stock solution of Cyclosporine A (Cys A) was prepared in MeOH (HPLC grade) and stored in an amber bottle to protect it from light. A solution of 0.10 mg/ml was used to prepare a calibration curve. A dilution series of the sample was prepared by using different amounts of Cys A. The standard Cys A and acetonitrile (HPLC grade) were obtained from Sigma Co. A calibration curve was created by using a dilution series of the standard Cys A.

Chromatographic analysis was performed using a HPLC Water Instrument (Lilford, MA, U.S.A.) model 60000A HPLC Pump, model 480 variable-wavelength UV detector, model 710B sample injector, and model 730 data module. A Waters RP-18 analytical column (30 cm X 4.6 mm I.D., particle size 5 mm), with a RP-18 guard column was heated to maintain the column at 70°C. The flow-rate of the mobile phase was 1ml/min and produced a pressure of 1100 p.s.i.

UV monitoring was conducted at 215 nm. The mobile phase was a mixture of degassed acetonitrile:water (65:35) in isocratic mode. The retention time for Cys A standard was 5.7 min.

Thin layer chromatography of standards and fungal extracts was performed using Silica Gel_{F254} as a stationary phase, and Chloroform:MeOH (97:3) as a mobile phase. TLC developed plates were visualized using iodine vapors and a 254 nm UV light source (Roesel and Kahan 1987). Cyclosporine A appears as a light yellow-brown spot under visible light and has an *Rf* value of 0.49 under the above conditions.

Amino Acid Analysis of Cordyceps sinensis

Ascomata of *Cordyceps sinensis* were obtained from North American Reishi Inc. Vancouver, although the sporocarps were originally collected in China. The fungus, including the colonized host, was submitted for amino acid analysis (see Appendix B, Figure B.3). Thirty grams of *Cordyceps sinensis* were separated into two parts: fungus ascocarps (15 g) and mummified caterpillar (15 g), which was not identified.

The two samples were extracted separately. Samples were ground with a mortar and a pestle, using liquid nitrogen. The pulverized material was transferred to 500-ml Erlenmeyer flasks, 200 ml of MeOH were added to each flask, and these were kept on a shaker for 24 hrs. The material was then filtered through Whatman No.1 paper and the filtrate was dried using a rotoevaporator. The concentrated crude extract was fractionated by passage through a 8.0 cm x 1.5 cm column packed with Dowex 50W-X8 mesh 20-50 cation exchange resins (JT Baker). The resin was first washed with deionized water, followed by 50 ml of a HCl solution pH 4.0, and finally 0.1 M NaOH. There were rinses of deionized water between each step. The column was then charged with 0.1 M HCl. The crude extract was dissolved in 10 ml of 50% MeOH and loaded into a resin column. The non-amino acid fraction was eluted with deionized water. The amino acids bound to the resin were eluted using 10% NH_4OH. Fifteen fractions were eluted (20 ml per fraction).

Samples from the collected fractions were dried to remove the NH_4OH, resuspended in MeOH and then spotted onto TLC cellulose plates. The plates were developed with Solvent A:Butanol-Water-Acetic acid (BWA) in a ratio of 45:20:20, and sprayed with 0.1% g of monohydrate ninhydrin (Sigma Co.) to determine which fractions contained similar amino acids. Amino acid containing fractions were pooled together and dried. The samples were refrigerated at 4°C. The amino acid-containing fraction (90 mg) was dissolved in 6 ml MeOH and separated by TLC using Cellulose FTLC Plastic sheets (EM Science 10x10) using a two-dimensional development system. Amino acids reacted with ninhydrin producing purple, violet, blue, and yellow colorations.

RESULTS

Biological Activity Assays: Antifungal, Antibiotic, and Phototoxic

I assessed the biological activities of some extracts from EF or the spent culture medium. Twenty crude fungal extracts of EF were active against the gram (+) bacteria *Staphylococcus aureus* and *Bacillus subtilis*. No activity was found against the gram (-) bacteria tested; only a strain of *Beauveria* sp. inhibited *E. coli* after UV light exposure.

A number of biological activities were detected from the extracts of EF (Table 3.1). The wide range of biological activity was obtained using an assortment of growing conditions (e.g., radiation with UV light). *Paecilomyces* sp. (85-14b) was the only strain that showed antifungal activity against the yeast *Candida albicans* (not shown in table). In total, seven fungal extracts were phototoxic against *Staphylococcus aureus* after exposure to ultraviolet light (350 nm). A total of 14 extracts showed antibacterial activity against one or more of the tested bacteria.

Table 3.1. Biological activity of entomogenous fungal extracts. Symbols: + = biological activity observed, ++ = biological activity only when irradiated with UV light, - = no biological activity observed. *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa* were used for anti-bacterial bioassays. Only *Paecilomyces* sp. (80-14a) showed moderate antifungal activity against *Candida albicans*.

	B. subtilis	*E. coli*	*S. aureus*	*P aeruginosa*
Control				
Methanol	-	-	-	-
Tetracycline	+	+	+	+
Fungi				
Beauveria bassiana(B1)	-	++	-	-
B. bassiana(B4)	++	++	-	-
B. bassiana(B6)	-	-	+	-
B. bassiana(B7)	-	-	+	-
Cordyceps capitata (84-20)	+	+	-	-
C. japonica(9647)	+	-	+	-
C. militaris(01-07)	+	-	-	-
C. militaris(5298)	+	-	-	-
C. ophioglosiodes(8992)	+	-	-	-
C. tuberculata(01-04)	-	-	-	-
Paecilomyces (84-2)	-	+	+	-
Paecilomyces(95-10)	+	-	-	-
Paecilomyces(24-2b)	-	-	++	-
Paecilomyces(98-2)	-	-	+	-
P. marquandii(80-14a)	+	+	+	+
Paecilomyces (80 -14b)	+	+	-	-
P. marquandii(73-21)	-	-	++	-
P. marquandii (95-2)	+	-	++	-
Verticillium sp. (100-2)	-	-	++	-
Verticillium sp. (100-1)	-	-	++	-

Photoactivated Antibiotics and Antifungal Substances

Four crude extracts of EF showed antibiotic activity against *Staphylococcus aureus*; one extract of *Beauveria bassiana* inhibited *E. coli*. The extracts were photoactivated against *S. aureus*. *Paecilomyces marquandii* (73-21 and 95-2), *Paecilomyces* spp (24-2b), and *Verticillium* sp. (100-1 and 100-2). The crude extract of *B. bassiana* (B-1) was phototoxic to *E. coli* after UV exposure.

Two dimensional TLC (2D-TLC) overlay bioassay was used to assay for antibacterial, antifungal, and phototoxic activity. The assay was also used as a guide for the fractionation of the crude extract of *Paecilomyces marquandii*.

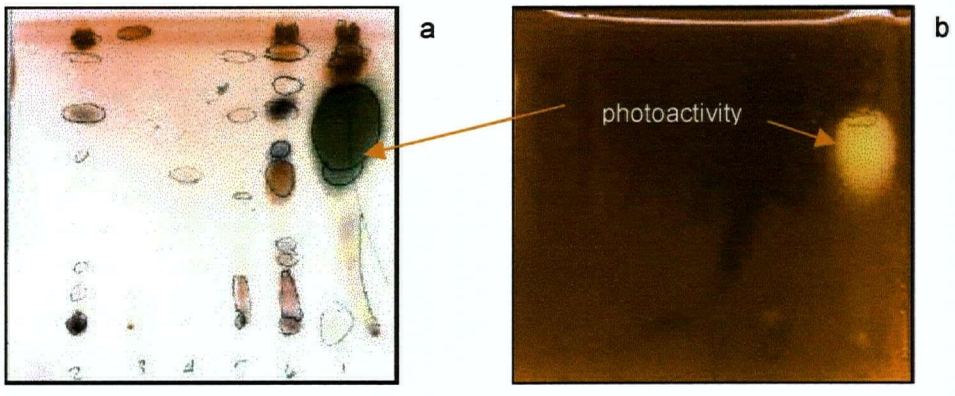


Figure 3.2. a) Chromatogram of fraction from an extract of *Paecilomyces marquandii,* TLC silica gel; b) duplicate plate used to bioassay for photoactivated antibiotics, positive test is indicated by the yellow area. The solvent system used to develop the TLC plate was: Chloroform: MeOH (9:1); the spray reagent was Vanillin.

A duplicate TLC plate was used for overlaid assay (Figure 3.3b), MH agar containing phenol red (MH-PR) was inoculated with *S. aureus*, poured on top of the TLC plate, allowed to solidify and incubated for 15 min. The replicate plate was irradiated with UV at 350 nm for 30 min, and then incubated for 18 hrs at 26°C. Spray reagent: MTT used to enhance the yellow area corresponding to the zone of phototoxicity.

The same technique is shown on a two-dimensional TLC (2D-TLC), testing a different *Paecilomyces* crude extract for antibacterial activity. This technique allowed a fast comparison of different extracts from related strains.

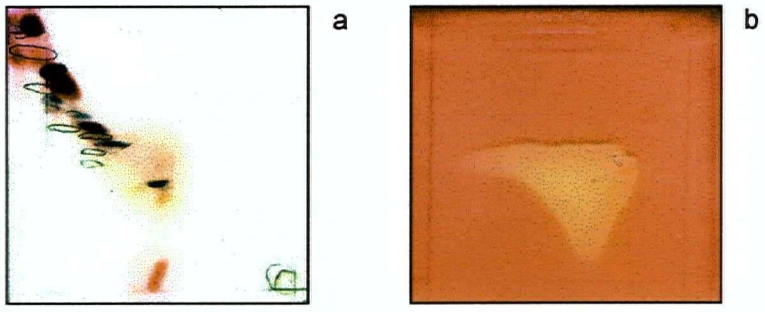


Figure 3.3. a) Two-dimensional chromatography of a crude extract of *Paecilomyces* sp. (strain 84-16a); spray reagent: vanillin, heating at 110°C until colors developed; b) duplicate TLC plate, without spraying, overlaid with HM+PR inoculated with *Staphylococcus aureus.* After 18-hr incubation at 26°C, plate was spayed with reagent MTT to visualize the area of inhibition (yellow area = antibacterial activity).

Antiviral Tests

Five of the tested EF extracts completely inhibited HSV-1 (Table 3.2). Two strains of *Paecilomyces* spp. showed antiviral activity without causing cytotoxic effects. The crude extracts of two other isolates obtained from *Torrubiella raticaudata*, and *T. mirabilis* Samson & Evans showed partial viral inhibition and were not cytotoxic. Complete cytotoxicity was exhibited by *Beauveria* sp. (B-7), *Cordyceps militaris* (01-07), *Paecilomyces tunuipes* (24-2b), *Torrubiella* sp. (01-06).

In the bioassay, the control consisted of Vero cells without the virus (Figure 3.4a). The cells with HSV-1 infection became smaller, round, and aggregated (Figure 3.4b). The effects of the cytotoxicity disrupt the organization of the Vero cells and causes membrane rupture and cell death (Figure 3.4c).

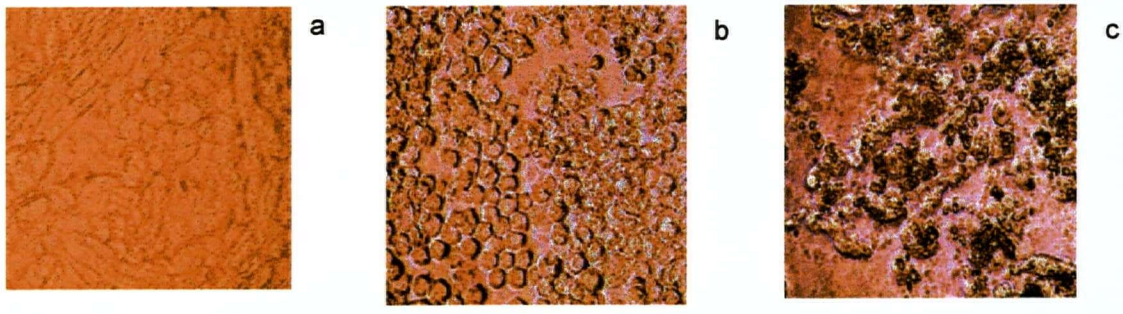


Figure 3.4. a) Healthy monkey kidney cells; b) monkey kidney cells showing HSV-1 viral infection, notice the shape of the cells, round and smaller than those in the control; c) monkey kidney cells showing the effects of a cytotoxic crude fungal extract, many kidney cells have collapsed or burst. Readings of the bioassay and photographs were taken after 72 hours of incubation.

Table 3.2. Antiviral bioassay. EtOaC extracts from entomogenous fungi were tested against herpes simplex virus (HSV-1). Toxicity test results: T = toxicity, PI = partial inhibition, CI = complete inhibition, and V = virus alive.

FUNGUS	Strain	200 µg/ml	100 µg/ml	50 µg/ml	25 µg/ml
Beauveria bassiana	B-3	V	V	V	V
Beauveria bassiana	WB	V	V	V	V
Beauveria bassiana	B-1	V	V	V	V
Beauveria sp.	B-7	**T**	**CI**	V	V
Cordyceps militaris	01-07	**T**	V	V	V
Paecilomyces sp.	95-2	**CI**	**PI**	V	V
Paecilomyces marquandii	73-21	V	V	V	V
Paecilomyces tenuipes	80-14a	**T**	**CI**	**CI**	V
Paecilomyces sp.	85-15	**CI**	V	V	V
Paecilomyces tenuipes	24-2b	**T**	**CI**	V	V
Torrubiella sp.	01-01	V	V	V	V
Torrubiella sp.	01-06	**T**	V	V	V
Torrubiella mirabilis	01-08	**PI**	V	V	V
Torrubiella raticaudata	01-09	**PI**	V	V	V
Verticillium sp	100-1	V	V	V	V
Verticillium sp.	Spi 2	V	V	V	V
Beauveria bassiana	Bet 4	T	T	PI	PI
Cordyceps militaris	5298	V	V	V	V
Cordyceps japonica	9647	V	V	V	V

Amino Acid Analysis of Cordyceps sinensis

The amino acid (AA) fraction of *Cordyceps sinensis* sporocarps and mummified caterpillars (extracted separately) were analyzed using an amino acid (AA) analyzer (Beck 3000r), Biotechnology, UBC (see Appendix B). The data from the AA analyzer were used to prepare a two-dimensional TLC (2D-TLC) standard mixture using commercially available AA (Sigma Co.) and the separated AA fraction from *C. sinensis*. Both chromatograms served as references suitable for preliminary comparison of the amino acid content in *Cordyceps* spp. and related groups.

The host caterpillar fraction was slightly different from the fruiting body of *Cordyceps sinensis*. All 18 standard amino acids used were detected by the AA analyzer, those in lower quantities were below 200 pmol, and most of these were not detected by TLC. The most abundant amino acids found in both caterpillars and sporocarps (amino acid analyzer; see Appendix B) were: glutamic acid, alanine, and proline; followed by valine, threonine, serine, phenylalanine, leucine, lysine, tyrosine, isoleucine, aspartic acid, histidine, arginine, and methionine. The purpose of this analysis was to identify potential chemo-taxonomical markers for *C. sinensis*. No rare or unique amino acids were detected from this analysis. Table 3.3 lists amino acids detected by TLC chromatography and comparison to true standards amino acids.

Table 3.3. R_f values for standard amino acids used in 2D-TLC analysis of *Cordyceps sinensis*. Solvent system A: Butanol-Water-Acetic Acid (BWA) in a ratio of 45:20:20; solvent system B: 1-Propanol- NH_4OH in a ratio of 55:45.

Amino Acid	R_f Value Sol A (BWA)	R_f Value Sol B
DL- Methionine	0.23	0.50
DL-Proline	0.13	0.27
D-Serine	0.04	0.26
Glycine	0.20	0.63
L-Alanine	0.01	0.07
L-Arginine	0.06	0.02
L-Glutamine	0.41	0.63
L-Histidine	0.09	0.33
L-Phenylalanine	0.16	0.38
L-Valine	0.27	0.49

A chromatogram was created using the same AA fraction analyzed in the AA analyzer (Figure 3.5). This chromatogram is a chemical fingerprint of the AA fraction of *C. sinensis* and could be compared with other *Cordyceps* spp. The Rf values were comparable to standard AA samples.

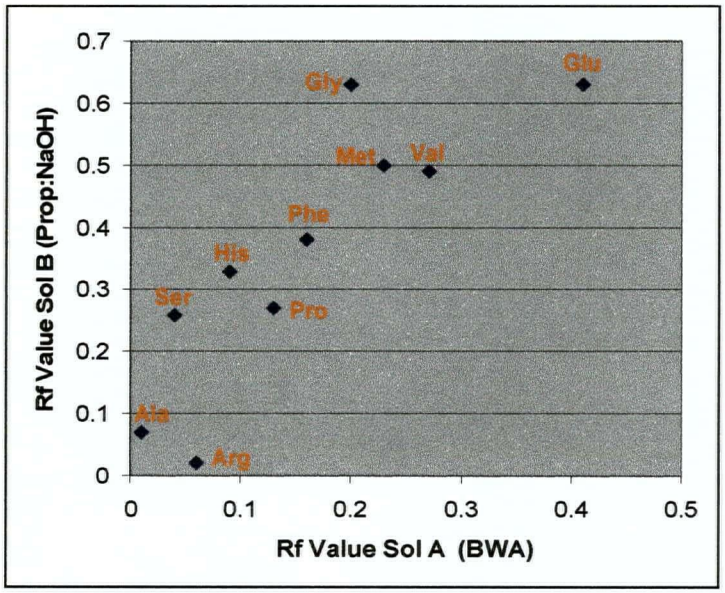


Figure 3.5. Amino acid analysis of *Cordyceps sinensis* using 2-Dimensional Thin Layer Chromatography. Points indicate position of amino acids on TLC plate. Solvent system: A) Butanol:Water:Acidic Acid (45:20:20); B) Propanol: NH_4OH (55:45).

Screening for Cyclosporine A

TLC and HPLC analyses of selected EF fungal extracts did not reveal the presence of Cyclosporine A.

DISCUSSION

Anamorphic stages seem to enjoy a higher diversity in morphological expression and changes in basic metabolic pathways. Little information is available on metabolic changes at declining stages of EF cultures. The detection of biologically active compounds also depends on an understanding of metabolic changes occurring over the life span of the fungal culture.

The EF extracts tested showed activity against four bacteria, one fungus, and one virus. Microorganisms used in the bioassays were selected for their relevance to human health. There was great variability in the results obtained in the screening. Variability within the same species is a well-known characteristic of fungal strains.

One of the challenges encountered during the search for antiviral drugs was cytotoxicity. Because viruses are obligatory parasites, the inhibition or elimination of a virus frequently interferes with the host cell chemistry and physiology. Partial inhibition of the viral infection by the EF crude extract may result from low concentrations of the antiviral compound. Cytotoxicity was exhibited by four of the EF species assayed for anti-viral activity. Cytotoxicity has also been previously correlated with antitumor activity, and cytotoxic compounds have been found to be potential anticancer agents. In future research, EF extracts exhibiting cytotoxicity should be tested against cancer cell lines.

I was unable to detect the presence of Cyclosporine A in crude extracts of EF tested, however this may require further investigation. Other Cyclosporines may have been present in the extracts, but no standards were available for comparison. The culture medium, growing conditions, and the stage of development at the time of extraction may require further modifications. The composition of the culture medium is perhaps the most important factor in the production of this cyclopeptide.

Cyclosporine A has immunosupressor activity in humans and is a useful drug for organ transplants. It is also effective in treating autoimmune disorders such as psoriasis and rheumatoid arthritis (Phillips 1991). Cyclosporine A was discovered as an antifungal agent produced by the fungus *Tolypocladium inflatum* Gams. This fungus was also known by the names *Trichoderma polysporum* (Link ex Pers.) (Dreyfuss 1976), and *Cylyndrocarpon lucidum* Booth (Borel 1986). *Tolypocladium inflatus* was renamed *Beauveria nivea*, and is considered the anamorph of *Cordyceps subsessilis* (Hodge *et al.* 1996) and *Cylindrocarpon lucidum* (Joug and Gantt 1987).

Sterol metabolism seems to be highly dependent on the quantity, quality, and diversity of lipids present in the substrate. Many filamentous fungi are also capable of bio-transforming endogenous compounds (Hoover 1993), a characteristic used for many years, and rapidly gaining popularity in the fields of biotechnology and industrial mycology. EF may be good candidates for the development of agents of biotransformation, and their biological activity could be improved by collection and screening of wild strains, or by manipulation of established fungal collections.

The biological potential of EF remains untapped in the BC coastal temperate rainforests. Future research on EF with biological activities should consider the modification of growing conditions and the determination of nutritional requirements. The production of metabolites of particular interest could be manipulated to enhance their yield. This may also allow EF to behave as agents of biotransformation.

CHAPTER FOUR: Evaluation of EF Tolerance to Heavy Metals

INTRODUCTION

Fungi are known to be efficient scavengers that sequester many organic and inorganic molecules. They are capable of survival in most environments and tolerate chemical toxicity, extreme temperature and pH, starvation, and dehydration. Heavy metals (HM) such as cobalt, copper, and nickel serve as micronutrients and are used by fungi and other organisms in redox processes, enzyme functionality, electrostatic interactions, and the regulation of osmotic pressure (Bruins *et al.* 2000). High levels of HM are toxic to most organisms, but some filamentous fungi are tolerant to moderate levels of HM toxicity (Zamani 1985).

Many filamentous fungi are capable of bio-transforming exogenous compounds (Hoover 1993), a characteristic highly appreciated and rapidly gaining popularity in the fields of biotechnology and industrial mycology. It has been suggested that as an adaptive strategy to HM toxicity, organisms generate small molecules (i.e. phenols, acidic amino acids) in large numbers on the cell surface to limit the uptake of the metals (Suresh and Subramanyan 1998). In addition, in the presence of toxic levels of heavy metals, sequestration of the heavy metals may occur (Mullen *et al.* 1992). EF have been isolated from soil samples using a selective medium containing copper (Baath 1991), however this medium is not 100% selective and other fungi are recovered as well. HM tolerance mechanisms used by EF remain poorly described.

Metal ion interactions occur mainly in the plasma membrane, which is made up of sterols, phospholipids, glycolipids (Weete 1973), proteins, and carbohydrates (Kendrik 1992). Toxic concentrations of metal ions can block functional groups in enzymes and transport systems, and displace essential metal ions in molecules and structural components, which may lead to increased membrane permeability and leakage of intracellular solutes (Gadd 1986).

Copper is essential for the activity of a number of physiologically important enzymes in many organisms, and is required in trace amounts. In humans, enzyme-related malfunctions may contribute to severe neurological symptoms and neurological disease. Copper is a co-

factor of Cu/Zn-superperoxidase-dismutase, which plays a role in the cellular response to oxidative stress by scavenging reactive oxygen species. In excess, *Cu++* inhibits sterol biosynthesis of the fungus symbiont in the lichen *Bryoria fuscescens* (Gyeln.) Brodo & Hawks (Tarhanen *et al.* 1999). This inhibition leads to potassium (K^+) efflux and a reduction in ergosterol concentration (Tarhanen *et al.* 1999).

Ergosterol is an essential lipid involved in growth, and in the regulation of permeability of cell membranes. Consequently, it is essential in the viability and health of fungal cells. Ergosterol is the main constituent and most abundant sterol in the cell membrane of fungi (Weete 1973). In the bi-layer structure of the membrane, ergosterol forms clusters within the phospholipid layers (Nagle and Tristam-Nagle 2000). Some antifungal drugs (e.g., Nystatin) act by binding to ergosterol in the membrane, or by inhibiting various enzymes along the biosynthetic mevalonate pathway leading to ergosterol (Kleinkauf and von Dohren 1988). Cerebrosides, sphingolipids (SPLs), ceramides, and phospholipids are also essential constituents of fungal cell membranes. Free fatty acids, and functional groups such as carboxyls, amines, hydroxyls, and thiols are present in the cell membrane (Gadd 1986).

Lanosterol is the precursor of animal sterols and also occurs in fungi. The antifungal compound fluconazole selectively inhibits lanosterol 14 α-dimethylase, a key enzyme in the maintenance of the fungal cell membrane. In *Candida albicans*, fluconazole was shown to produce a profound depletion of ergosterol with a significant increase in lanosterol content (Pearce 1995). The enzyme, 3-oxidosqualene-lanosterol cyclase (OSC) is involved in the biosynthesis of ergosterol (Jolidon *et al.* 1997) and could be a target for the development of new antifungal drugs.

A substantial amount of research has focussed on fungi as sources of compounds useful in controlling mammalian steroid chemistry (Capek *et al.* 1996*a*; Gao *et al.* 2001*a*; Nam *et al.* 2001). Some of the most potent and clinically useful compounds as cholesterol metabolism effectors have been derived from fungal products. Two examples are hydroxymethyl coenzyme A reductase (HMG-CoA reductase) from *Penicillium brevicompactum*, and lovastatin from

Aspergillus terreus (Alberts *et al.* 1967). *Paecilomyces* sp. has been used in steroid biotransformation (Capek *et al.* 1996) and members of this genus may be potential candidates for the biotransformation of other toxic metabolites and mixtures of toxic waste (e.g., industrial residues).

Toxic metals interact with essential cellular components through covalent and ionic bonding (Bruins *et al.* 2000). Cadmium, lead, and mercury selectively concentrate in certain fungi (Michelot *et al.* 1999). Some of the metal resistance mechanisms reported in microorganisms include: a) exclusion by permeability barrier, b) intra- and extra-cellular sequestration, c) active transport efflux pumps, d) enzymatic detoxification, and e) reduction in the sensitivity of cellular targets to metal ions (Bruins *et al.* 2000). EF may use one or more of these mechanisms to deal with HM toxicity.

I evaluated the tolerance of EF to HM, and assessed the effects of HM on fungal growth and morphology. I also isolated and characterized a cerebroside with antibacterial activity. This antibiotic was produced when *Paecilomyces marquandii* was grown on a medium containing copper sulphate. My main interest was to gain understanding of EF responses to heavy metals, in particular changes in biological activities due to the presence of heavy metals in the culture medium.

METHODS

Preparation of Fungal Cultures

A conidial suspension of *Paecilomyces marquandii was* prepared from 10-day-old petri plate cultures (MYP). The plates were flooded with a solution of 0.05% (v/v) Tween 80. A 100-ml sample of the conidial suspension was taken in a dilution series to give a final concentration of 2×10^6 spores/ml. One half ml of the final suspension was used to inoculate each Erlenmeyer flask. Flasks contained 500 ml of MYP broth, and were incubated at $25 \pm 4^{\circ}C$, with cycles of 12/12 hr L/D using grow lights (Sylvania). Cultures were kept under these conditions for 21 days. They were then harvested, and EtoAC extracts were prepared.

Samples for HPLC analysis were prepared from cultures grown in petri plates. The basic medium used was ¼ strength for all ingredients of MYP (Bandoni *et al.* 1970), with the addition of Cu_2SO_4. For the preparation of solid media containing HM, the pH was adjusted to 8.5; an alternative was to double the amount of agar in the medium. Concentrations of HM were prepared at 100, 200, 300, 400, 500, and 1000 µg/L. Pb, Zn, Co, Fe, and Ni were used to assess tolerance to other HM. The effects of HM on EF growth and morphology were recorded, and some were assessed chemically.

Comparison of Fungal Extracts by TLC and HPLC

Ethyl acetate extracts from the liquid medium were compared using TLC. The extract was applied to a 10x10-cm silica Gel $_{(F254)}$ plate and run in CH_2Cl_2:MeOH (9:1) as the moving phase. Plates were air dried, examined under long and short wave UV, sprayed with vanillin, and heated to 110°C until pigmentation was visible. *Rf* values were established for major compounds and compared to standard samples of cyclosporin, ergosterol, lanosterol, and ergosterol peroxide.

Fractionation of Crude Extracts

Twenty-five liters of liquid MYP containing 400 mg $CuSO_4$/liter of medium were inoculated with a spore suspension (prepared as above). Cultures were maintained stationary, at room temperature for 21 days: two weeks in the dark and one week of 12/12 hr L/D using grow lights (Sylvania). The mycelium was then harvested by filtering through Whatman No. 1 filter paper, gently rinsing three times with distilled water, and storing at -4°C overnight. The frozen material was thawed, mixed with sea sand, triturated in a mortar, and extracted with 500 ml of EtoAc:MeOH (2:1 v/v) for five hrs at room temperature. An additional extraction was performed using 100% EtoAc.

Extracts were combined, reduced in volume using a rotary evaporator, and then the residue was extracted three times with equal volumes of EtOAc. The EtOAc extract was

fractionated by gel filtration using a Silica Gel column (230-400 mesh) and eluted with a gradient of Hexane:Acetone (Figure 4.1). The liquid medium control and filtered spent medium were extracted with non-polar organic solvents (e.g., Hexane and EtOAc), reduced under pressure, as above, and stored at 4°C. Twenty-five individual fractions were separated from the extract and compared by TLC (Silica GF_{254}, solvent system: Cyclohexane:Acetone 50:50). These fractions were assessed for antibacterial, antifungal, and phototoxic activity.

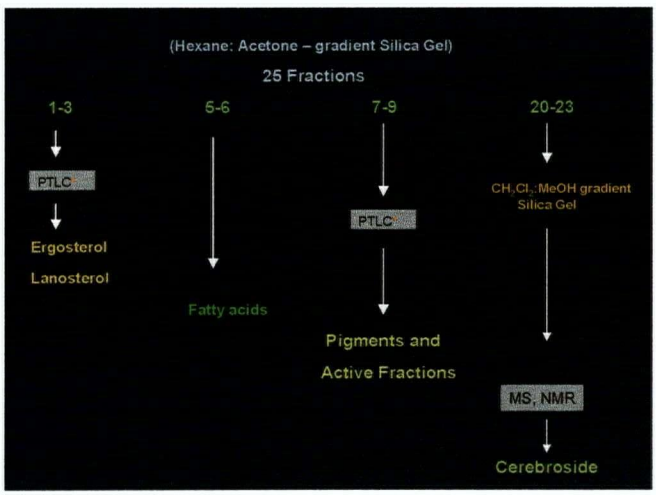


Figure 4.1. Fractionation scheme of the crude extract *Paecilomyces marquandii*.
TLC, UV light, and color spot reactions using vanillin, molybdenic acid, and ninhydrin reagents detected different classes of compounds.

Further purification of the active fraction 7-9 (Figure 4.1) was performed by using standard 20x20 cm PTLC plates (E. Merck, GF_{254}) coated with a 0.25-mm-thick layer of silica. The PTLC plates were developed using a solvent system containing cyclohexane:acetone (50:50) v/v. Fractions obtained from PTLC plates were extracted in chloroform, concentrated under vacuum, examined by TLC silica plates GF_{254}, and developed using the above solvent system. Plates were air-dried, examined under short and long wave UV, sprayed with vanillin reagent, and heated in an oven at 110°C until pigmentation developed. Molybdenic acid was also used as a detection reagent. The developed plates were immersed in a 5% methanolic solution of molybdenic acid, followed by heating as above.

Fractions 20-23 (Figure 4.1) were pooled and further fractionated by PTLC (Silica GF_{254}, with cyclohexane:acetone (50-50) as the solvent system) to yield 6.0 mg of compound 1, which gave a reddish-purple color with vanillin reagent, and deep blue with a pale greenish-yellow background with molybdenic acid.

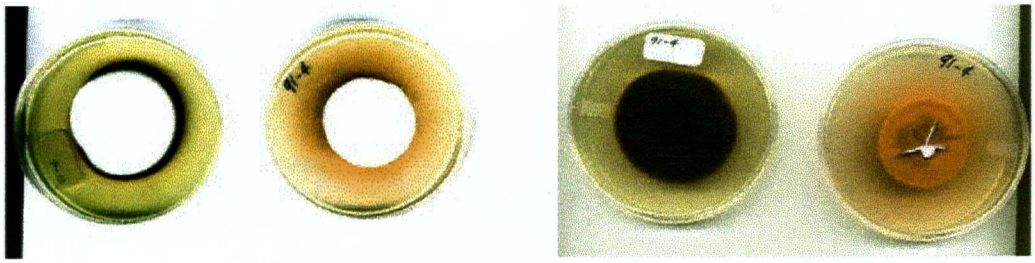
Isolated pure compounds were submitted to MS, ^{1}H-NMR, ^{13}C-NMR, COSY (^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY), and HMBC for structural determination. Data are included in Appendix B, Figure B.4. Fractions 1-3 (Figure 4.1) yielded an unidentified fatty acid; fractions 5-6 contained ergosterol and lanosterol; fractions 7-9 yielded an antibiotic and several pigments; fractions 10-15 contained a combination of unidentified compounds.

RESULTS

Effects of Heavy Metals on Entomogenous Fungi

Fungal Growth and Morphology

A decrease in growth and changes in pigment coloration were observed in most EF species. A *Paecilomyces marquandii* control plate showed yellow mycelia, a color intensifying with age (Figure 4.2). This pigmentation changed to reddish-orange in the medium containing HM. The reddish-orange pigmentation appearing in the underside of the mycelia mat (Figure 4.2, Lower surface B) was produced in response to HM presence. Colonies of *P. marquandii* growing in solid and liquid MYP-Fe medium produced an orange pigment on the lower surface of the colony. Some pigments reacted positively to the reagent 1,1-Diphenyl-2picrylhydrazine (DPPH; a free radical).

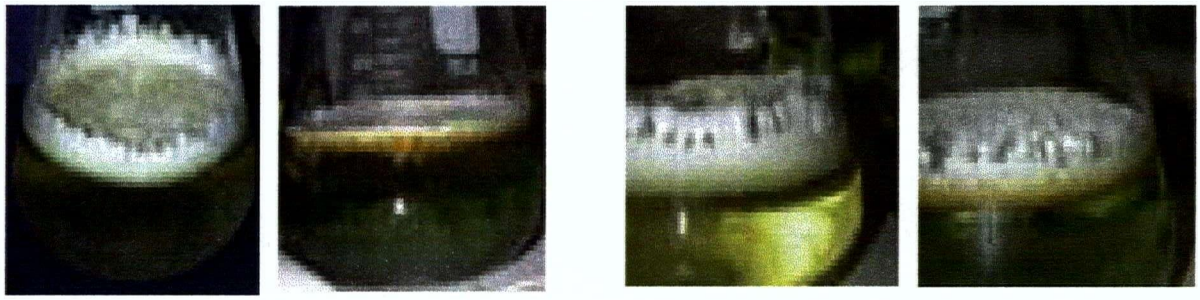


A B A B

Upper surface *Lower surface*

Figure 4.2. Colonies of *Paecilomyces* sp. (strain 91-4) growing on MYP medium containing heavy metals. A = copper; B = copper and iron.

HM caused a general reduction in EF growth (Figure 4.3) and biomass (Figure 4.4). The effect of pH and the addition of copper and iron to the medium is illustrated in Figure 4.4. Growth under copper conditions was assessed for *C. militaris*. Growth was reduced by 50% in HM treatments. Pigmentation decreased in the presence of copper and iron at pH 9.0, but not in the presence of iron pH 7.0 (Figure 4.5).



A B C D

Figure 4.3. *Paecilomyces marquandii* growing in media containing iron (B) and copper (D). A and C are controls.

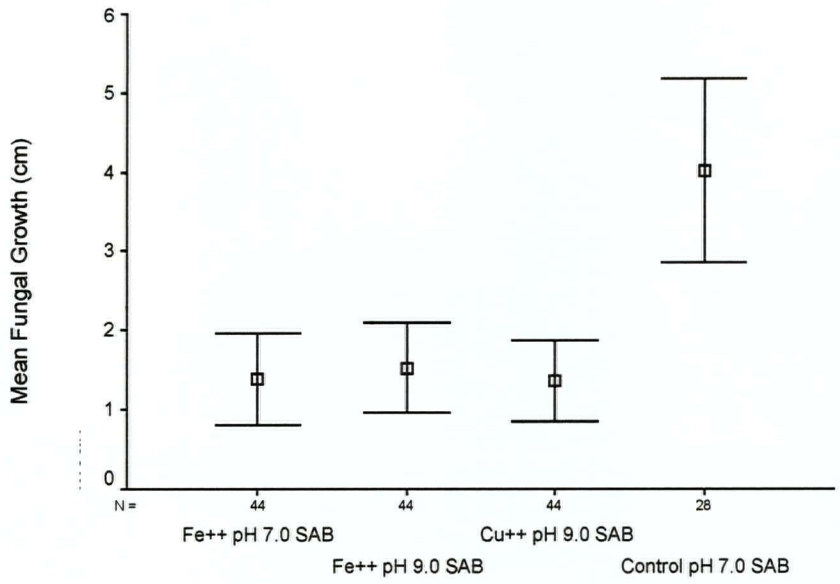


Figure 4.4. Influence of iron and copper at different pH values on *Cordyceps militaris* growth rate. Growth was measured in centimeters. Error bars indicate 2 standard errors of the mean.

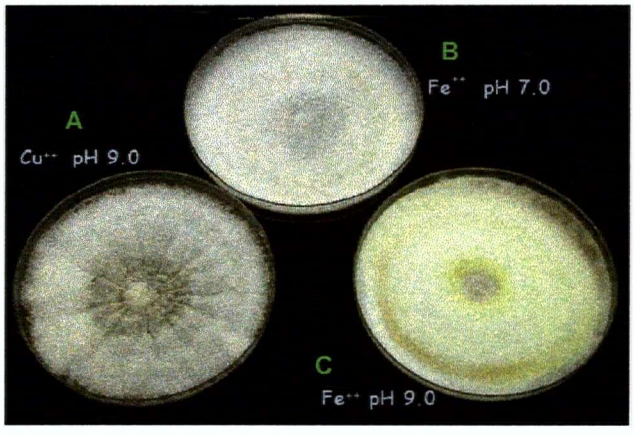


Figure 4.5. Growth of *Cordyceps militaris* 01-07 on media containing copper at A) pH 9.0, B) iron at pH 7.0, and C) pH 9.0. Heavy metal concentration: 500 µg/L of medium (MYP). Cultures were 6 days old.

Figure 4.6 illustrates the TLC of the chloroform fraction of crude extract samples of *Cordyceps militaris* (01-07- pH: 7.0; 01-07-pH:9.0; and 01-07-pH 6.0-control), *Cordyceps japonica* 9647, Media (MYP), and control. The solvent system used for TLC was Chloroform:MeOH 9:1. Figure 4.7 illustrates a TLC of EtoAc extracts of selected EF.

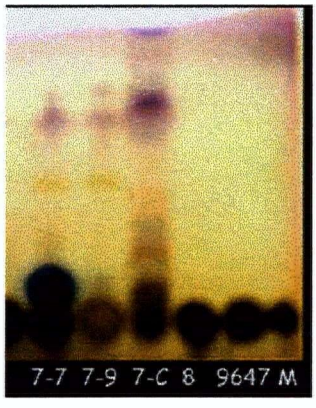


Figure 4.6. TLC showing the accumulation of intermediate or complex metabolites produced by EF growing in an iron enriched medium, 7-7, 7-9, and 7-C correspond to *Cordyceps militaris* (01-07) growing on iron media at pH 7.0 and pH 9.0; *Cordyceps japonica* 9647; and M corresponds to the medium (MYP); and 8 corresponds to an isolate of *Torrubiella* sp. Spray reagent: Vanillin-sulphuric acid and heating. Note the blue spot at the bottom of chromatogram; sample 7-7 growing under iron conditions at pH 7.0. The spot is absent at pH 9.0.

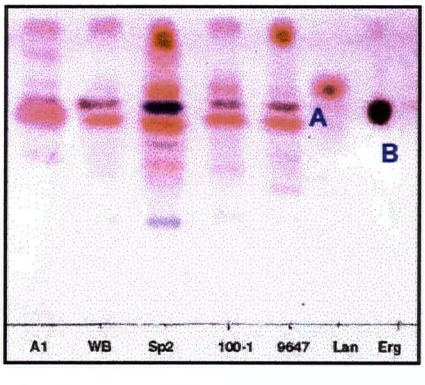


Figure 4.7. Thin layer chromatogram of EtOaC extracts of selected entomogenous fungi. Lanosterol (A) and ergosterol (B) standards are shown.

Fungal Tolerance to Heavy Metals

Tolerance to HM varied among EF strains of the same fungus, and among genera (Table 4.1). Sterol content and pigmentation also varied. The most toxic HM to EF was copper, followed by iron, nickel, lead and cobalt. Many isolated strains of *Paecilomyces* were tolerant to copper and other HM (Table 4.1). A strain of *Paecilomyces* spp. 85-15a was capable of growing in a medium containing 1g Cu++/L.

Table 4.1. Selected entomogenous fungi growing in media containing heavy metals. Concentration was 500 µg/L of MYP medium. Y = growth under the particular metal.

Fungus/Heavy Metal	**Copper**	**Zinc**	**Lead**	**Nickel**
Paecilomyces marquandii (73-21)	Y	Y	Y	
Paecilomyces sp. (85-14)		Y	Y	Y
Paecilomyces sp. (92-4)		Y	Y	
Paecilomyces sp. (95-2)		Y	Y	
Paecilomyces marquandii (85-15)	Y	Y	Y	
Paecilomyces sp. (98-2)		Y	Y	
Beauveria bassiana (5711)		Y		
Cordyceps ophioglosiodes (8992)	Y	Y		
Cordyceps militaris (0107)	Y	Y	Y	
Cordyceps japonica (9647)		Y		
Beauveria bassiana (8554)		Y		Y

EF growing on MYP medium containing HM are shown in Figure 4.8. Three strains of *Paecilomyces* showed similar tolerance to lead (Figure 4.8A). Notice the effect of copper in the formation of concentric rings in *Paecilomyces* sp. 98-2 (Figure 4.8B, lower right plate). *Epichloë festuca* was tolerant to HM toxicity, but its growth was repressed.

P. tunuipes 96-21 ---- *P. marquandii* 73-21

Pb

Zn ---- Ni

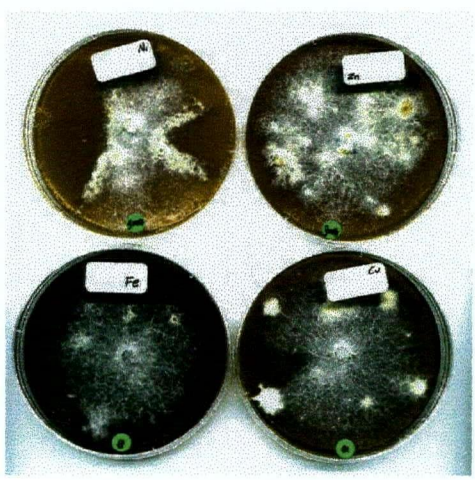
A *Paecilomyces* sp. 84-14a

B *Paecilomyces* sp. 98-2

Fe ---- Cu

Ni ---- Zn

Fe --- Cu



C *Epichloë fustuca* 96-10

Figure 4.8. Entomogenous fungi growing on MYP medium containing heavy metals. A) *Paecilomyces tunuipes*, B) *Paecilomyces* sp., C) A member of the Clavicipitaceae, *Epichloë festuca*, a relative of *Cordyceps* sp., is symbiotic in temperate grasses.

Paecilomyces marquandii was tolerant to all HM tested. The presence of Cu^{++} in the media resulted in a reduction of ergosterol production. TLC showed the accumulation or overproduction of different compounds. Results of a cerebroside isolated from *P. marquandii* are reported in the following section. Two other antibiotic compounds were detected during the extract analysis of *P. marquandii*, however the amounts isolated were too small to fully characterize these compounds.

Bioactive Compounds Produced by EF Growing under HM Conditions

From the initial screening, the isolate *Paecilomyces marquandii* (73-21) was selected for its fast growth rate and resistance to copper toxicity. The biomass produced by this strain under copper conditions allowed for the characterization of a cerebroside.

Isolation of a Cerebroside

I isolated the cerebroside 4E-8E)-N-2-hydroxyhexadecanoyl-1-O-β-glucanopyranosyl-9-methyl-C^{18}-sphinga-4,8-diene (Figure 4.9), which was produced by *Paecilomyces marquandii* when growing in MYP broth containing copper sulphate. This cerebroside may be produced by *P. marquandii* in the absence of copper, but in such small quantities, it was not detected. Under copper conditions, the cerebroside became one of the most abundant compounds in the chloroform fraction of the crude extract.

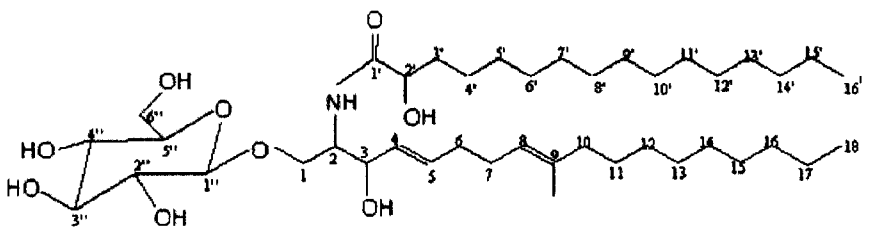


Figure 4.9. Chemical structure of compound 1: (4E,8E)-N-2-hydroxyhexadecanoyl-1-O-β-glucanopyranosyl-9-methyl-C^{18}-sphinga-4,8-diene, a cerebroside.

The cerebroside (Figure 4.9) is an amorphous powder. EIMS m/z: 709 (M^+-H_2O, 3), 576 (6), 547 (9), 531 (5), 516 (11), 500(3), 474 (3), 438 (2), 368 (20), 296 (20), 276 (30), 262 (15), 225 (8), 180 (10), 97 (60), 95 (40), 83 (60), 81 (35), 69 (85), 57 (60), 55 (100); FABMS m/z 750 (M^++Na), 728 (M^++H), 710 (MH-H_2O); ^{1}H- NMR (400MHz, CD_3OD): δ 3.70 (1H, *m*, H-1a), 4.11 (1H, *m*, H-1b), 3.97 (1H, *m*, H-2), 4.12 (1H, *m*, H-3), 5.49 (1H, *m*, H-4), 5.75 (1H, *m*, H-5), 2.05 (2H, *m*, H-6), 2.04 (2H, *m*, H-7), 5.14 (1H, *m*, H-8), 1.98 (2H, *t*, *J* 7.3, H-10), 1.41 (2H, *m*, H-11), 1.25 (12H, *m*, H-12 to H-17), 0.89 (3H, *t*, *J* 7.0, H-18), 1.61 (3H, *s*), 4.01 (1H, *m*, H-2′), 1.54 (1H, *m*, H-3′a), 1.69 (1H, *m*, H-3′b), 1.40 (2H, *m*, H-4′), 1.20-1.35 (22H, *m*, H-5′ to H-15′), 0.89 (3H, *t*, *J* 7.0, H-16′), 4.26 (1H, *d*, *J*, 7.8, H-2″), 3.18 (1H, *dd*, *J* 7.8, 9.1, H-3″), 3.26 (1H, *m*, H-4″), 3.25 (1H, *m*, H-5″), 3.83 (1H, *dd*, *J* 12.0, 1.8, H-6″a), 3.63 (1H, *dd*, *J* 12.0, 4.3, H-6″b);^{13}C-NMR (100MHz, CD_3OD): δ 69.7 (C-1), 54.6 (C-2), 72.9 (C-3), 131.1 (C-4), 134.7 (C-5), 35.9 (C-6), 28.7 (C-7), 124.8 (C-8), 136.8 (C-9), 40.8 (C-10), 29.1 (C-11), 23.7-33.1 (6C, C-12 to C-17), 14.5 (C-18), 16.1 (C-19), 177.2 (C-1′), 73.1 (C-2′), 35.9 (C-3′), 26.2 (C-4′), 30.2-30.8 (11C, C-5′ to C-15′), 14.5 (C-16′), 104.7 (C-1″), 75.0 (C-2″), 77.9 (C-3″), 78.0 (C-4″), 71.6 (C-5″), 62.7 (C-6″).

Ergosterol and lanosterol were also isolated and their identities confirmed by MS and comparison with pure standards (Appendix B, Figure B.4). Included are: a) low resolution FAB Mass Spectrum, b) ^{13}C-NMR Spectrum, c) ^{1}H-^{1}H COSY Spectrum, and d) ^{1}H-NMR Spectrum.

Antimicrobial Activity of a Cerebroside

The cerebroside (4E,8E)-N-2-hydroxyhexadecanoyl-1-O-β-glucanopyranosyl-9-methyl-C^{18}-sphinga-4,8-diene, was identified as one of the main components of the crude fungal extract. TLC of the cerebroside illustrates antibacterial activity against a methicillin resistant strain of *Staphylococcus aureus* (Figure 4.10). No activity was found in phototoxicity, antiviral, and antifungal bioassays.

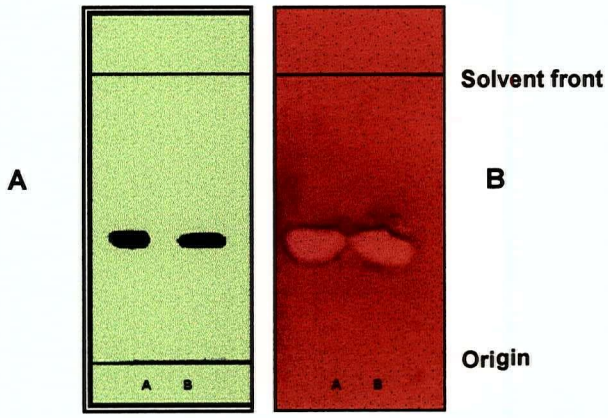


Figure 4.10. Thin Layer Chromatography illustrating Compound 1: (4E,8E)-N-2-hydroxyhexadecanoyl-1-O-β-glucanopyranosyl-9-methyl-C^{18}-sphinga-4,8-diene. Solvent system: Cyclohexane: acetone 50:50 (v/v). A). Compound 1 spotted twice on plates and gave an intense blue color reaction with molybdenic acid. B) Replicate plate, overlaid antibacterial bioassay; organism: *Staphylococcus aureus.*

DISCUSSION

Effects of HM on EF Growth, Morphology, and Tolerance

The production of free radical scavengers may contribute to HM tolerance. This suggests that pigments may interact with HM as antioxidants, binding to HM as chelator molecules. Pigments produced by *P. marquandii* may act as antioxidants or chelating agents to reduce copper toxicity. TLC and HPLC analysis suggests that copper toxicity affects ergosterol and lanosterol production. Copper may also contribute to the accumulation of intermediates and/or *de novo* production of sterols, sphingolipids, ceramides, phospholipids, and fatty acids.

Morphological and biochemical effects of HM were demonstrated in my preliminary experiments. In general, anamorph strains were more tolerant to HM than teleomorph strains (i.e. Osaka strains). HM affected EF in different ways. Zinc may enhance pigment and conidia production, leading to restricted growth of *Cordyceps* spp. and *Beauveria* spp., more so than *Paecilomyces* spp. HPLC analyses of *in vivo* copper induction suggest that yellow pigments and ergosterol in *Paecilomyces* sp. and *Cordyceps militaris* may play a role in the formation of HM-complexes. *Cordyceps* spp. are less tolerant to the HM tested, but more analyses are

needed to provide an explanation for this response. Some species of EF showed resistance or tolerance to high concentrations of heavy metals, however the mechanisms of metal resistance have not been elucidated for this group.

Filamentous fungi may play a role in the *in situ* biodegradation of aliphatic pollutants in the soil (April *et al.* 2000). I found that many species of EF exhibited resistance or tolerance to copper, iron, nickel, iron, lead, and cobalt. In other organisms, toxic concentrations of these metal ions can block functional groups in enzymes and transport systems, and displace essential metal ions in molecules and structural cell components (Gadd 1993). This leads to increased membrane permeability and leakage of intracellular solutes.

EF growth rate was affected by the presence of HM in the culture media, but this may not be what happens in nature. TLC of crude extracts suggests that copper affects ergosterol production, and possibly contributes to the accumulation of intermediates and/or to the *de novo* production of other lipids. Steroid transformation *in vitro has* been shown to be a feature of some species of *Paecilomyces* (Capek *et al.* 1976). It is reasonable to assume that one mechanism of tolerance used by *Paecilomyces* sp. is the sequestration of metal ions (e.g., copper).

Neurospora crassa immobilizes copper by binding it to phenols present in the mycelial cell wall. The accumulation of copper in the cell wall gives the mycelia its bluish color (Lerch 1991). Metallothioneins are a family of important metal binding proteins involved in the handling of trace metals, including HM. A gene coding metallothioneins in *Neurospora crassa* has been reported (Müenguer and Lerch 1987) and expressed in *Escherichia coli*. The recombinant *E. coli* producing the metallothionein protein was able to accumulate large amounts of cadmium, suggesting a potential use for metallothionein-based bioabsorbent for certain HM removal applications (Pazirandeh *et al.* 1995). This technique could be used in the removal of HM from aqueous media (Pazirandeh and Campbell 1998) and some EF may be good candidates for bioremediation research and applications.

Nickel toxicity has been linked to carcinogenicity and multiple types of cellular damage, which ultimately results in altered gene expression, rather than indirect DNA damage (Zoroddu *et al.* 2001). Iron is an essential trace element, abundant in nature, and involved in many metabolic and anabolic processes. Iron participates in diverse pathological processes by catalyzing the formation of reactive oxygen free radicals (Chau 2000). In addition, microorganisms excrete a variety of high affinity, low molecular-weight (500-1500) iron sequestering agents called siderophores (Wong 1983; Jalal *et al.* 1984). Fungal siderophores include ferrichromes, coprogens (Wong 1989), and triacetylfusarinine C (Konetschny-Rapp *et al.* 1988). A new class of siderophores, rhizopherrin, which employs neither hydroxamate nor catecholate groups, has been isolated in the zygomycetes (Drechsel *et al.* 1991, 1992).

Bioactive Compounds Produced by EF under HM Conditions

The cerebroside, 4E-8E)-N-2-hydroxyhexadecanoyl-1-O-β-glucanopyranosyl-9-methyl-C^{18}-sphinga-4,8-diene, was originally discovered from the imperfect fungus, *Fusicossum amygdali* (Ballio *et al.* 1979). Other sources have been described in the last two decades, including *Polyporus ellisii* (Gao *et al.* 2001) and *Termitomycetes albuminosus* (Berk.) Heim. (Qi *et al.* 2001).

Recently two cerebrosides with antifungal activity were reported from *Russula achroleuca* (Gao *et al.* 2001). Two ceramides with C18 phytosphingosides have been isolated from *R. cyanoxantha* (Gao *et al.* 2001) and *Armillaria mellea* (Gao *et al.* 2001). A new glucocerebroside was recently described from the mushroom *Polyporus ellissi* Berk (Gao *et al.* 2001). Conclusive evidence has been shown that the ceramide hydroxyl groups are involved in linkages with proteins (Steward *et al.* 2001). The hydroxyl groups of sugar could possibly participate in ester linkages.

The main compound isolated from *P. marquandii* was identified as (4E-8E)-N-2-hydroxyhexadecanoyl-1-O-β-glucanopyranosyl-9-methyl-C^{18}-sphinga-4,8-diene. Further research is required to elucidate the changes occurring in fungal metabolism and membrane

rearrangement under HM stress. The increase or decrease in the production of steroids in response to HM may be a fungal defence against stress and/or a toxicity tolerance mechanism.

Studies of dry mycelia have shown that certain fungi have a large sorption capacity (SC). For example, *Aspergillus terreus* dry mycelium has a high SC of 224 µg/g dry wt, and the mycelia become blue with increased copper in the medium (Gulati 1999). Large amounts of biomass waste results from some fermentation processes using *Aspergillus* spp., *Penicillium* spp. and other filamentous fungi. The mycelia by-products are not utilized and are generally disposed of as garbage. Some species of *Paecilomyces* may be good candidates for dry mycelia removal of HM as they showed fast growth rate and high biomass production under HM conditions, and they can be grown in different substrates at low cost.

Detection of larger amounts of the cerebroside may indicate changes in lipid biosynthesis. Mycelia color changed from yellow to a greenish-yellow suggesting copper deposition in the cell wall of *P. marquandii*. In liquid medium, *P. marquandii* developed an orange-brownish ring on the lower side of the colony. These pigments may be acting as chelating or antioxidant agents to counter balance HM presence.

The modification of sterols by free radical scavengers is suggested as a mechanism of biotransformation or immobilization of HM by EF. Many microorganisms are known to produce compounds with surface-active characteristics generally termed "biosurfactants." These are amphilic molecules containing both lipids and hydrophobic moiety and include glycosphingolipids and gangliosides (Isoda *et al.* 1997).

HM may interact with a variety of ligands such as carboxyl, phosphate, and amino groups. Under different pH levels several complexes of copper, iron, nickel and other HM may form. Changes in the structural arrangement of the lipid bilayer of membranes can influence the functional properties of membrane bound and peripheral proteins. These play an important role in morphogenesis and normal tissue remodelling through their interactions with the cytoskeleton (Baka 2000).

Reduced fungal growth observed under copper treatment could be the result of changes in the cell membrane's lipid composition and arrangement. The characterization of mixtures and single lipids may answer some of the questions of lipid bilayers in membranes. At present, there are many uncertainties in the literature (Nagle and Tritam-Nagle 2000).

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APPENDICES

Appendix A

Table A.1. List of entomogenous fungi collected in British Columbia.

Collection No.	Host/Substrate	Collection Date	Place
Paecilomyces marquandii 73-21	beetle	Feb 98	UBC
Paecilomyces sp. 73-22	beetle	Feb 98	UBC
Paecilomyces sp. 73-23	White fly	March 98	UBC
Verticillium sp. 80-2	Spider	June 98	Squamish
Beauveria bassiana 80-3	Beetle parts	June 98	Squamish
Beauveria bassiana 80-4	Beetle	June 98	Squamish
Torrubiella sp. 80-15	Spider	June 98	Cypress Bowl
Paecilomyces sp. 81-13	Beetle larvae	August 98	Capilano GVRD
Beauveria bassiana 81-14	Adult beetle	August 98	Capilano GVRD
Paecilomyces sp. 84-2	Beetle	October 98	Capilano GVRD
Paecilomyces marquandii 85-14	Caterpillar	October 98	Capilano GVRD
Torrubiella sp. 85-13	Spider	October 98	Capilano GVRD
Beauveria bassiana 91-20	CWD smear	February 99	Ladner
Beauveria bassiana 91-21	CWD smear	February 99	Ladner
Paecilomyces inflatus 94-1	Small larvae	April 99	UBC
Paecilomyces marquandii 95-2	Beetle larvae	May 99	Capilano GVR
Paecilomyces sp. 96-10	Beetle larvae	May 99	UBC
Epichlöe festuca 96-12	Grass	June 99	UBC
Gibellula sp. 96-21	Spider	June 99	Squamish
Paecilomyces sp. 98-2	Beetle larvae	July 99	UBC
Gibellula sp. 98-3	Spider	July 99	UBC
Beauveria bassiana 98-15	Beetle larvae	July 99	Squamish
Paecilomyces marquandii 98-18	Beetle larvae	July 99	Squamish
Gibellula pulchra 100-1	Spider	Oct 99	Vancouver Island

Table A.2. Media used in different experiments for culturing entomogenous fungi.

Malt extract-Yeast extract- Peptone (MYP):

Malt extract	14.00 g
Yeast extract	0.25 g
Peptone	0.5 g
Water	1000 ml

Dissolve the solids in water, add the agar and autoclave the medium for 15 min at 121°C. Note: 1/4 of the malt in the full recipe for MYP (Bandoni 1972) media was used for the initial isolation. Tetracycline was added at a rate of 1µg/l to suppress bacterial growth.

Malt Agar:

Malt extract	20 g
Agar	15 g
Water	1000 ml

Dissolve the malt extract in water; add the agar and autoclave the medium for 15 min at 121°C.

Potato Dextrose Agar (PDA):

Dissolve 36 grams of Difco PDA (pre-mixed) in 1000 ml of water and autoclave the medium for 15 min at 121°C.

Oatmeal Agar:

Rolled oats	30 g
Agar	15 g
Water	1000 ml

Cook oatmeal in water for 15-30 min in a container over boiling water (double boiler). Filter through three or four layers of cheesecloth and bring filtrate to one liter of water. Autoclave for 15 min. at 121°C.

Czapek (Dox) Agar:

Sodium nitrate (Na NO3)	20 g
Potassium phosphate (K2HPO4)	1.0 g
Potassium chloride (KCl)	0.5 g
Magnesium sulphate (MgSO4.7H2O)	0.5 g
Ferrous sulphate (FeSO4)	0.01 g
Sucrose	30.0 g
Water	1000.0 ml

Autoclave for 15 min. at 121°C.

Table A.2. Media used in different experiments for culturing entomogenous fungi. [*continued*]

Water Agar (Plain Agar, Non-nutrient Agar):

Agar (Difco)	15 g
Distilled water	1000 ml

Sterilize for 15 min at 121^{0}C.

Peanut and Walnut Media:

Twenty grams of peanuts and/or walnuts were placed in glass Petri dishes containing 0.5 ml of water. The dishes were autoclaved for 15 min at 121°C. Two-three pieces, 5 g, of sterile peanuts and/or walnuts were placed on the surface of previously prepared water agar plates. The plates were sealed with plastic wrap to allow exchange of gasses, placed in open plastic bags, and refrigerated at 4°C until further use.

Phenol Red Media (Over-laid Plate Bioassay):

The following amounts have been estimated for a petri plate:

Bacterial Media	
Muller Hinton Broth (powder)	0.42 g
Agar	0.12 g
Phenol red	0.8 ml
DH_2O	19.0 ml

Fungi Media	
SAB broth (powder)	0.72 g
Agar	0.12 9
Phenol red	0.8 ml
DH_2O	19.0 ml

The phenol stock solution used in the media was prepared to a concentration of 0.5 µg/ml. The amount of medium in the plate can be reduced to 10-15 ml.

Stains Used:

Phloxine Staining Solution
Phloxine was prepared as a 2% aqueous solution.

KOH Staining Solution
5% aqueous solution

Lactophenol Cotton Blue

Phenol crystals	20 g
Lactic Acid	20 ml
Glycerol	40 ml
Distilled water	20 ml

Dissolved by heating gently under a hot water tap. Add 0.05 g. of cotton blue.

Table A.3. Reported biological activities and other characteristics of *Paecilomyces marquandii*.

Reported biological activities and other characteristics *Paecilomyces marquandii*	**Literature cited**
Ovicidal and ovistatic	Basualdo *et al.* 2000
Human pathogen in patients with compromised immune systems	Naldi *et al.* 2000
Nematicidal activity	Che *et al. 1999*
Nematicidal activity	Esnard *et al.* 1999
Highly resistant to fluconazole and flucytosine	Guarro 1998
Nematicidal activity	Chen *et al.* 1996
Hyalohyphomycotic agents	Sekhon *et al.* 1996
Nonapeptide antibiotic, cytotoxic, and phytotoxic	Dosio *et al.* 1994
Nematicidal activity	Marban *et al.* 1992
Keratinolytic, keratinophilic	Filipello *et al.* 1991
Nematicidal activity	Walter *et al.* 1990
Resistant to amphotericin B and 5-fluorocytosine, but sensitive to imidazoles	Castro *et al.* 1990
Resistant to amphotericin B and 5-fluorocytosine, but sensitive to imidazoles	Maslem *et al.* 1988
Mycosis in fish	Lightner *et al.* 1988
Peptide antibiotic	Radics *et al.* 1987
Peptide antibiotic	Rossi *et al.* 1987
Member of the fungal winter community during the colder periods of the year.	Widden 1986
Mycoparasite	Van Der Aa 1986
Ovicidal activity	Lysek 1985

Appendix B

Figure B.1 DNA sequences of entomogenous fungi producing significant alignments using GenBank: a) *Verticillium* sp. 100-1, b) *Paecilomyces tenuipes* 24-2b, c) *Beauveria bassiana* Bet-1, d) *Cordyceps militaris* 01-07, e) *Paecilomyces* sp. 85-15, f) *Paecilomyces* sp. 85-2, g) *Paecilomyces marquandii* 73-21. The sequences were compared using blast analysis against *Neurospora crassa* genome (Whitehead Institute) other analysis still pending.

Figure B.2 Chromatograms of free fatty acids from selected entomogenous fungi analyzed by gas chromatography. Vial number as indicated in chromatogram report: 1 = *Beauvaria bassiana* Bet-3; 2 = *Paecilomyces* sp. 85-14; 3 = *Verticillium* sp. 100-1; 4 = *Paecilomyces* sp. 95-2; 5 = *Cordyceps militaris* 01-07; 6 = *Paecilomyces tenuipes* 24-2b; 7 = *Paecilomyces marquandii* 73-21; 11 = solvent control; 12 = fatty acid standards

Figure B.3 Chromatograms of Amino acid analysis from sporocarps of *Cordyceps cyanensis*. Included are: Chromatogram Report, Mol Percent Report, and Typical Amino Acid Analysis Results (Hydrolysis Test Peptide).

Figure B.4 Physicochemical data used to determine the structure of the cerebroside (4E,8E)-N-2-hydroxyhexadecanoyl-1-O-β-glucanopyranosyl-9-methyl-C^{18}-sphinga-4,8-diene. Included are: a) low resolution FAB Mass Spectrum, b) ^{13}C-NMR Spectrum, c) ^{1}H-^{1}H COSY Spectrum, d) ^{1}H-NMR Spectrum.

Figure B.1. DNA sequences of entomogenous fungi producing significant alignments using GenBank: a) *Cordyceps militaris* 01-07, b)*Verticillium* sp. 100-1, c) *Paecilomyces tenuipes* 24-2b, d) *Paecilomyces marquandii* 73-21, e) *Paecilomyces* sp. 85-15, f) *Paecilomyces* sp. 85-2, and g) *Beauveria bassiana* Beet-1. The sequences were compared using blast analysis against *Neurospora crassa* genome (Whitehead Institute).

Partial nucleotide sequence of 18s rDNA of *Cordyceps militaris* (01-07).

GTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCAATTATACAGC
GAAACTGCGAATGGCTCATTATATAAGTTATCGTTTATTTGATAGTACCTTACTACTTGGATA
ACCGTGGTAATTCTAGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGTATTTAT
TAGATTAAAAACCAATGCCCTCTGGGCTCCTTGGTGATTCATGATAACTCTTCGAATCGCAC
GGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTTCGATGTTTGGGTAT
TGGCCAAACATGGTTGCAACGGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAGC
CTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGAT
TCGGGGAGGTAGTGACAATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATG
AGTACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCG
CGGTAATTCCAGCTCCATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAAC
CTTGGGCCTGGCTGGCCGGTCCGCCTCACCGCGTGCACTGGTCCGGCCGGGCCTTTCCC
TCTGTGGAACCCCATGCCCTTCACTGGGCGTGGCGGGGAAACAGGACTTTTACTTTGAAA
AAATTAGAGTGCTCCAGGCAGGCCTATGCTCGAATACATTAGCATGGAATAATGAAATAGG
ACGCGCGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGG
GGCATCAGTATTCAACGGTCAGAGGTGAAATTCTTGAATTCCTTGAAGACTAACTACTGCG
AAAGCATTCGCCAAGGATGTTTTCATTAATCAGGAACGAAAGTTAGGGGATCGAAGACGAT
CAGATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGACGATGTTATTTTT
TGACGCGTTCGGCACCTTACGAGAAATCAAAGTGCTTGGGCTCCAGGGGGAGTATGGTCG
CAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGGGTAAACTCTATATGCA
GCCGCAGTAGCTCTGCTCCGAAAAGCAGCCTGAAAGGGTTAATGGTGTTCCTGACCGCCG
CCCGGCGGTCGAATAATTGCTAGTCTCTTCGGAGGCGACACCCTCAAGTTGCGGGAACGG
CACGTGCGAACGTACGTGTGCCTTTAGAGCTGGCGCTACCAAGCAGGCGTGGAAAGCGC
GTCTGCGGCCGGGGTAATGACCTAGGGTATGGTAAAAACCCGTCAGATTAGGCAATCCGC
ATCCAAGCCCCGTCGCCGCAAGGCACGGGGAAGGATCAGAGACTTGACGGGGGTGGGTA
GCGGCGCACGCTGCCTAAGATAAAGTCCGACTGCTCGCGAAAGCGTGTCAGATAGGTAAC
CTATAATCGGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCGCCAGGTCCAGA
CACAATGAGGATTGACAGATTGAGAGCTCCTTCTTGATTTTGTGGGTGGTGGTGCATGGCC
GTTTTTAGTTGGTGGAGTGATTTGTCTGCTTAATTGCGATAACGAACGAGACCTTGACCTG
CTAAATAGCCCGTATTGCTTTGGCAGTACGCTGGCTTCTTAGAGGGACCATCGGTGCAATC
CGAAGGAAGTTCGAGGCAAAAACAGGTCTGTAATGCCCTTAGATGTTCTGGGCCGCACGC
GCGCTACACTGACGGAGCCAGCGAGTTCTTCCTTGTCCGAAAGGTCCGGGTAATCTTGTT
AAACTCCGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAACGAGGAATCCCTAG
TAAGCGCAAGTCATCTGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCG
CTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACT
ACCACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC
AAGGTCTCCGTAGGTGAACCTGC.

Table B.1a. Accession number and score for the 10 most similar EF deduced from partial sequence of *Cordyceps militaris* (01-07), using BLAST search of Genebank sequences.

Rank	Accession Number	Organism	Probability, Score (bits)
1	AB027334	*Paecilomyces tenuipes*	2192
2	AB044630	*Cordyceps* sp. 97005	2165
3	AB044629	*Cordyceps pruinosa*	2151
4	AB027335	*Beauveria brongniartii*	2141
5	AF280633	*Paecilomyces tenuipes*	2137
6	D85136	*Cordyceps militaris*	2135
7	AB027333	*Cordyceps takaomontana*	2123
8	AB044631	*Claviceps africana*	2111
9	AF281132	*Cordyceps* sp. 97009	2097
10	AB032475	*Paecilomyces fumoso-roseus*	2089

Partial nucleotide sequence of 18s rDNA of *Verticillium* sp. (100-1).

GTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCAATTATACAGC
GAAGACTGCGAATGGCTCATTATATAAGTTACCGTTTATTTGATAGTACCTTACTACTTGGA
TAACCGTGGTAATTCTAGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGTATTT
ATTAGATTAAAAACCAATGCCCTCTGGGCTCCTTGGTGATTCATAATAACTTTTCGAATCGC
ATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTTCGATGTTGGGTA
TTGGCCAAACATGGTCGCAACGGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAG
CCTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGA
TTCGGGGAGGTAGTGACAATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAAT
GAGTACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCC
GCGGTAATTCCAGCTCCATAGCGTATATAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAAC
CTTGGGCCTGGCTGGCCGGTCCGCCTCACCGCGTGTACTGGTCCGGCCGGGCCTTTCCC
TCTGTGGAACCTCATGCCCTTCACTGGGTGTGGCGGGGAAACAGGACTTTTACTTTGAAAA
AATTAGAGTGCTCCAGGCAGGCCTATGCTCGAATACATTAGCATGGAATAATGAAATAGGA
CGTGTGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTTATGATTAATAGGGACAGTCGGGG
GCATCAGTATTCAATTGTCAGAGGTGAAATTCTTGGATTTATTGAAGACTAACTACTGCGAA
AGCATTTGCCAAGGATGTTTTCATTAATCAGGAACGAAAGTTAGGGGATCGAAGACGATCA
GATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGACGATGTTATTTTTTG
ACGCGTTCGGCACCTTACGAGAAATCAAAGTGCTTGGGCTCCAGGGGGAGTATGGTCGCA
AGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGGGTGGAGCCTGCGGCTTAA
TTTGACTCAACACGGGGAAACTCACCAGGTCCAGACACAATGAGGATTGACAGATTGAGA
GCTCTTTCTTGATTTTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGT
CTGCTTAATTGCGATAACGAACGAGACCTTAACCTGCTAAATAGCCCGTATTGCTTTGGCA
GTACGCCGGCTTCTTAGAGGGACTATCGGCTCAAGCCGATGGAAGTTTGAGGCAATAACA
GGTCTGTAATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGA
GTACTTCCTTGTCCGAAAGGCCCGGGTAATCTTGTTAAACTCCGTCGTGCTGGGGATAGAG
CATTGCAATTATTGCTCTTCAACGAGGAATCCCTAGTAAGCGCAAGTCATCTGCTTGCGTT
GATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTG
AGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACCACCCAGGGCCGGAAAGCTCTCC
AAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC

Table B.1b. Accession number and score for the ten most similar fungi deduced from partial sequence of *Verticillium* sp. (100-1), using BLAST search of Genebank sequences.

Rank	Accession Number	Organism	Probability, Score (bits)
1	AB0273334	*Paecilomyces tenuipes*	2192
2	AB044630	*Cordyceps* sp. 97005	2165
3	AB044629	*Cordyceps pruinosa*	2165
4	AB027335	*Beauveria brongniartii*	2151
5	AF280633	*Beauveria bassiana*	2141
6	D85136	*Paecilomyces tenuipes*	2137
7	AB027333	*Cordyceps militaris*	2135
8	AB044631	*Cordyceps takaomontana*	2123
9	AF281176	*Claviceps africana*	2111
10	AB027332	*Cordyceps* sp. 97009	2097

Partial nucleotide sequence of 18s rDNA of *Paecilomyces tunuipes* 24-2B.

GTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCAATTATACAGC
GAAACTGCGAATGGCTCATTATATAAGTTATCGTTTATTTGATAGTACCTTACTACTTGGATA
ACCGTGGTAATTCTAGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGTATTTAT
TAGATTAAAAACCAATGCCCTCTGGGCTCCTTGGTGATTCATAATAACTTTTCGAATCGCAT
GGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTTCGATGTCTGGGTA
TTGGCCAAACATGGTCGCAACGGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAG
CCTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGA
TTCGGGGAGGTAGTGACAATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAAT
GAGTACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCC
GCGGTAATTCCAGCTCCATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAA
CCTTGGGCCTGGCTGGCCGGTCCGCCTCACCGCGTGTACTGGTCCGGCCGGGCCTTTCC
CTCTGTGGAACCTCATGCCCTTCACTGGGTGTGGCGGGGAAACAGGACTTTTACTTTGAAA
AAATTAGAGTGCTCCAGGCAGGCCTATGCTCGAATACATTAGCATGGAATAATGAAATAGG
ACGTGTGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGG
GGCATCAGTATTCAATTGTCAGAGGTGAAATTCTTGGATTTATTGAAGACTAACTACTGCGA
AAGCATTTGCCAAGGATGTTTTCATTAATCAGGAACGAAAGTTAGGGGATCGAAGACGATC
AGATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGACGATGTTATTTTTT
GACGCGTTCGGCACCTTACGAGAAATCAAAGTGCTTGGGCTCCAGGGGGAGTATGGTCGC
AAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGGGTGGAGCCTGCGGCTTA
ATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACACAATGAGGATTGACAGATTGAGA
GCTCTTTCTTGATTTTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGT
CTGCTTAATTGCGATAACGAACGAGACCTTAACCTGCTAAATAGCCCGTATTGCTTTGGCA
GTACGCCGGCTTCTTAGAGGGACTATCGGCTCAAGCCGATGGAAGTTTGAGGCAATAACA
GGTCTGTAATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGA
GTACTTCCTTGTCCGAAAGGCCCGGGTAATCTTGTTAAACTCCGTCGTGCTGGGGATAGAG
CATTGCAATTATTGCTCTTCAACGAGGAATCCCTAGTAAGCGCAAGTCATCTGCTTGCGTT
GATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTG
AGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACCACCCAGGGCCGGAAAGCTCTCC
AAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC

Table B.1c. Accession number and score for the ten most similar fungi deduced from partial sequence of *Paecilomyces tunuipes* (24-2b), using BLAST search of Genebank sequences.

Rank	Accession Number	Organism	Probability, Score (bits)
1	AB003951	*Tritirachium* sp.	3336
2	AJ301994	*Myrothecium roridum*	3275
3	AJ301993	*Myrothecium roridum*	3275
4	AJ301995	*Myrothecium roridum*	3259
5	AB003949	*Nectria cinnabarina*	3253
6	AJ302005	*Myrothecium inundatum*	3251
7	AJ302000	*Myrothecium leucotrich*	3251
8	D85136	*Paecilomyces tenuipes*	3245
9	AJ302003	*Myrothecium verrucaria*	3243
10	AJ302002	*Myrothecium atroviride*	3243

Partial nucleotide sequence of 18s rDNA of *Paecilomyces marquandii* (73-21).

GTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCAATTATACAGC
GAAACTGCGAATGGCTCATTATATAAGTTATCGTTTATTTGATAGTACCTTACTACTTGGATA
ACCGTGGTAATTCTAGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGTATTTAT
TAGATTAAAAACCAATGCCCTCTGGGCTCCTTGGTGATTCATAATAACTTTTCGAATCGCAT
GGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTTCGATGTTTGGGTAT
TGGCCAAACATGGTCGCAACGGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAGC
CTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGAT
TCGGGGAGGTAGTGACAATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATG
AGTACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCG
CGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAA
CCTTGGGCCTGGCTGGCCGGTCCGCCTCACCGCGTGTACTGGTCCGGCCGGGCCTTTCC
CTCTGTGGAACCTCATGCCCTTCACTGGGTGTGGCGGGGAAACAGGACTTTTACTTTGAAA
AAATTAGAGTGCTCCAGGCAGGCCTATGCTCGAATACATTAGCATGGAATAATGAAATAGG
ACGTGTGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGG
GGCATCAGTATTCAATTGTCAGAGGTGAAATTCTTGGATTTATTGAAGACTAACTACTGCGA
AAGCATTTGCCAAGGATGTTTTCATTAATCAGGAACGAAAGTTAGGGGATCGAAGACGATC
AGATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGACGATGTTATTTTTT
GACGCGTTCGGCACCTTACGAGAAATCAAAGTGCTTGGGCTCCAGGGGGAGTATGGTCGC
AAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGGGTGGAGCCTGCGGCTTA
ATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACACAATGAGGATTGACAGATTGAGA
GCTCTTTCTTGATTTTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGT
CTGCTTAATTGCGATAACGAACGAGACCTTAACCTGCTAAATAGCCCGTATTGCTTTGGCA
GTACGCCGGCTTCTTAGAGGGACTATCGGCTCAAGCCGATGGAAGTTTGAGGCAATAACA
GGTCTGTAATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGA
GTACTTCCTTGTCCGAAAGGCCCGGGTAATCTTGTTAAACTCCGTCGTGCTGGGGATAGAG
CATTGCAATTATTGCTCTTCAACGAGGAATCCCTAGTAAGCGCAAGTCATCTGCTTGCGTT
GATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTG
AGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACCACCCAGGGCCGGAAAGCTCTCC
AAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC

Table B.1d. Accession number and score for the ten most similar fungi deduced from partial sequence of *Paecilomyces marquandii* (73-21), using BLAST search of Genebank sequences.

Rank	Accession Number	Organism	Probability, Score (bits)
1	AB003951	*Tritirachium* sp.	3360
2	AJ301994	*Myrothecium roridum*	3299
3	AJ301993	*Myrothecium roridum*	3299
4	AJ301995	*Myrothecium roridum*	3283
5	AB003949	*Nectria cinnabarina*	3277
6	AJ302005	*Myrothecium inundatum*	3275
7	AJ302000	*Myrothecium leucotrich*	3275
8	D85136	*Paecilomyces tenuipes*	3269
9	AJ302003	*Myrothecium verrucaria*	3267
10	AJ302002	*Myrothecium atroviride*	3267

Partial nucleotide sequence of 18s rDNA of *Paecilomyces* sp. (85-15).

GTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCAATTATACAGC
GAAACTGCGAATGGCTCATTATATAAGTTATCGTTTATTTGATAGTACCTTACTACTTGGATA
ACCGTGGTAATTCTAGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGTATTTAT
TAGATTAAAAACCAATGCCCTCTGGGCTCCTTGGTGATTCATAATAACTTTTCGAATCGCAT
GGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTTCGATGTTTGGGTAT
TGGCCAAACATGGTCGCAACGGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAGC
CTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGAT
TCGGGGAGGTAGTGACAATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATG
AGTACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCG
CGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAA
CCTTGGGCCTGGCTGGCCGGTCCGCCTCACCGCGTGTACTGGTCCGGCCGGGCCTTTCC
CTCTGTGGAACCTCATGCCCTTCACTGGGTGTGGCGGGGAAACAGGACTTTTACTTTGAAA
AAATTAGAGTGCTCCAGGCAGGCCTATGCTCGAATACATTAGCATGGAATAATGAAATAGG
ACGTGTGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGG
GGCATCAGTATTCAATTGTCAGAGGTGAAATTCTTGGATTTATTGAAGACTAACTACTGCGA
AAGCATTTGCCAAGGATGTTTTCATTAATCAGGAACGAAAGTTAGGGAATCGAAGACGATC
AGATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGACGATGTTATTTTTT
GACGCGTTCGGCACCTTACGAGAAATCAAAGTGCTTGGGCTCCAGGGGGAGTATGGTCGC
AAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGGGTGGAGCCTGCGGCTTA
ATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACACAATGAGGATTGACAGATTGAGA
GCTCTTTCTTGATTTTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGT
CTGCTTAATTGCGATAACGAACGAGACCTTAACCTGCTAAATAGCCCGTATTGCTTTGGCA
GTACGCCGGCTTCTTAGAGGGACTATCGGCTCAAGCCGATGGAAGTTTGAGGCAATAACA
GGTCTGTAATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGA
GTACTTCCTTGTCCGAAAGGCCCGGGTAATCTTGTTAAACTCCGTCGTGCTGGGGATAGAG
CATTGCAATTATTGCTCTTCAACGAGGAATCCCTAGTAAGCGCAAGTCATCTGCTTGCGTT
GATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTG
AGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACCACCCAGGGCCGGAAAGCTCTCC
AAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC

Table B.1e. Accession number and score for the ten most similar fungi deduced from partial sequence of *Paecilomyces* sp. (85-15), using BLAST search of Genebank sequences.

Rank	Accession Number	Organism	Probability, Score (bits)
1	AB003951	*Tritirachium* sp.	3252
2	AJ301994	*Myrothecium roridum*	3291
3	AJ301993	*Myrothecium roridum*	3291
4	AJ301995	*Myrothecium roridum*	3275
5	AB003949	*Nectria cinnabarina*	3269
6	AJ302005	*Myrothecium inundatum*	3267
7	AJ302000	*Myrothecium leucotrich*	3267
8	D85136	*Paecilomyces tenuipes*	3261
9	AJ302003	*Myrothecium verrucaria*	3259
10	AJ302002	*Myrothecium atroviride*	3259

Partial nucleotide sequence of 18s rDNA of *Paecilomyces* (85-2).

GTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCAATTATACAGC
GAAACTGCGAATGGCTCATTATATAAGTTATCGTTTATTTGATAGTACCTTACTACTTGGATA
ACCGTGGTAATTCTAGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGTATTTAT
TAGATTAAAAACCAATGCCCTCTGGGCTCCTTGGTGATTCATAATAACTTTTCGAATCGCAT
GGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTTCGATGTTTGGGTAT
TGGCCAAACATGGTCGCAACGGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAGC
CTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGAT
TCGGGGAGGTAGTGACAATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATG
AGTACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCG
CGGTAATTCCAGCTCCATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAAC
CTTGGGCCTGGCTGGCCGGTCCGCCTCACCGCGTGTACTGGTCCGGCCGGGCCTTTCCC
TCTGTGGAACCTCATGCCCTTCACTGGGTGTGGCGGGGAAACAGGACTTTTACTTTGAAAA
AATTAGAGTGCTCCAGGCAGGCCTATGCTCGAATACATTAGCATGGAATAATGAAATAGGA
CGTGTGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGGG
GCATCAGTATTCAATTGTCAGAGGTGAAATTCTTGGATTTATTGAAGACTAACTACTGCGAA
AGCATTTGCCAAGGATGTTTTCATTAATCAGGAACGAAAGTTAGGGGATCGAAGACGATCA
GATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGACGATGTTATTTTTTG
ACGCGTTCGGCACCTTACGAGAAATCAAAGTGCTTGGGCTCCAGGGGGAGTATGGTCGCA
AGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGGGTGGAGCCTGCGGCTTAA
TTTGACTCAACACGGGGAAACTCACCAGGTCCAGACACAATGAGGATTGACAGATTGAGA
GCTCTTTCTTGATTTTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGT
CTGCTTAATTGCGATAACGAACGAGACCTTAACCTGCTAAATAGCCCGTATTGCTTTGGCA
GTACGCCGGCTTCTTAGAGGGACTATCGGCTCAAGCCGATGGAAGTTTGAGGCAATAACA
GGTCTGTAATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGA
GTACTTCCTTGTCCGAAAGGCCCGGGTAATCTTGTTAAACTCCGTCGTGCTGGGGATAGAG
CATTGCAATTATTGCTCTTCAACGAGGAATCCCTAGTAAGCGCAAGTCATCTGCTTGCGTT
GATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTG
AGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACCACCCAGGGCCGGAAAGCTCTCC
AAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTGGGTGAACCTGC

Table B.1f. Accession number and score for the ten most similar fungi deduced from partial sequence of *Paecilomyces* sp. (85-2), using BLAST search of GenBank sequences.

Rank	Accession Number	Organism	Probability, Score (bits)
1	AB003951	*Tritirachium* sp.	3244
2	AJ301994	*Myrothecium roridum*	3283
3	AJ301993	*Myrothecium roridum*	3283
4	AJ301995	*Myrothecium roridum*	3267
5	AB003949	*Nectria cinnabarina*	3261
6	AJ302005	*Myrothecium inundatum*	3259
7	AJ302000	*Myrothecium leucotrich*	3259
8	D85136	*Paecilomyces tenuipes*	3253
9	AJ302003	*Myrothecium verrucaria*	3251
10	AJ302002	*Myrothecium atroviride*	3251

Partial nucleotide sequence of 18s rDNA of beet-1.

GTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCAATTATACAGC
GAAACTGCGAATGGCTCATTATATAAGTTATCGTTTATTTGATAGTACCTTACTACTTGGATA
ACCGNGGTAATTCTAGAGCTAATACATGCTGAAAATCCCGACTTCGGAAGGGATGTATTTA
TTAGATTAAAAACCAATGCCCTCTGGGCTCCTTGGTGATTCATAATAACTTTTCGAATCGCA
CGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCTCTATCAACTTTCGATGTTTGGGTA
TTGGCCAAACATGGTCGCAACGGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAG
CCTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGA
TTCGGGGAGGTAGTGACAATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAAT
GAGTACAATTTAAATCTCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCG
CGGTAATTCCAGCTCCATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAAC
CTTGGGCCTGGCTGGCCGGTCCGCCTCACCGCGTGTACTGGTCCGGCCGGGCCTTTCCC
TCTGTGGAACCTCATGCCCTTCACTGGGTGTGGCGGGGAAACAGGACTTTTACTTTGAAAA
AATTAGAGTGCTCCAGGCAGGCCTATGCTCGAATACATTAGCATGGAATAATAAAATAGGA
CGTGTGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGGG
GCATCAGTATTCAATTGTCAGAGGTGAAATTCTTGGATTTATTGAAGACTAACTACTGCGAA
AGCATTTGCCAAGGATGTTTTCATTAATCAGGAACGAAAGTTAGGGGATCGAAGACGATCA
GATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGACGATGTTATTTTTTG
ACGCGTTCGGCACCTTACGAGAAATCAAAGTGCTTGGGCTCCAGGGGGAGTATGGTCGCA
AGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGGGTGGAGCCTGCGGCTTAA
TTTGACTCAACACGGGGAAACTCACCAGGTCCAGACACAATGAGGATTGACAGATTGAGA
GCTCTTTCTTGATTTTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGT
CTGCTTAATTGCGATAACGAACGAGACCTTAACCTGCTAAATAGCCTGTATTGCTTTGGCA
GTACGCCGGCTTCTTAGAGGGACTATCGGCTCAAGCCGATGGAAGTTTGAGGCAATAACA
GGTCTGTAATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGA
GTACTTCCTTGTCCGAAAGGCCCGGGTAATCTTGTGAAACTCCGTCGTGCTGGGGATAGA
GCATTGCAATTATTGCTCTTCAACGAGGAATCCCTAGTAAGCGCAAGTCATCTGCTTGCGT
TGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGT
GAGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACCACCCAGGGCCGGAAAGCTCTC
CAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC

Table B.1g. Accession number and score for the ten most similar fungi deduced from partial sequence of *beet-1*, using BLAST search of GenBank sequences.

Rank	Accession Number	Organism	Probability, Score (bits)
1	AB003951	*Tritirachium* sp.	3214
2	AJ301994	Myrothecium roridum	3237
3	AJ301993	*Myrothecium roridum*	3237
4	AB003949	*Nectria cinnabarina*	3223
5	AJ301995	*Myrothecium roridum*	3221
6	AJ302005	*Myrothecium inundatum*	3213
7	AJ302000	*Myrothecium leucotrich*	3213
8	D85136	*Paecilomyces tenuipes*	3207
9	AJ302003	*Myrothecium verrucaria*	3205
10	AJ302002	*Myrothecium atroviride*	3205

Figure B.2. Chromatograms of free fatty acids from selected entomogenous fungi analyzed by gas chromatography. Vial number as indicated in chromatogram report: 1 = *Beauvaria bassiana* Bet-3; 2 = *Paecilomyces* sp. 85-14; 3 = *Verticillium* sp. 100-1; 4 = *Paecilomyces* sp. 95-2; 5 = *Cordyceps militaris* 01-07; 6 = *Paecilomyces tenuipes* 24-2b; 7 = *Paecilomyces marquandii* 73-21; 11 = solvent control; 12 = fatty acid standards

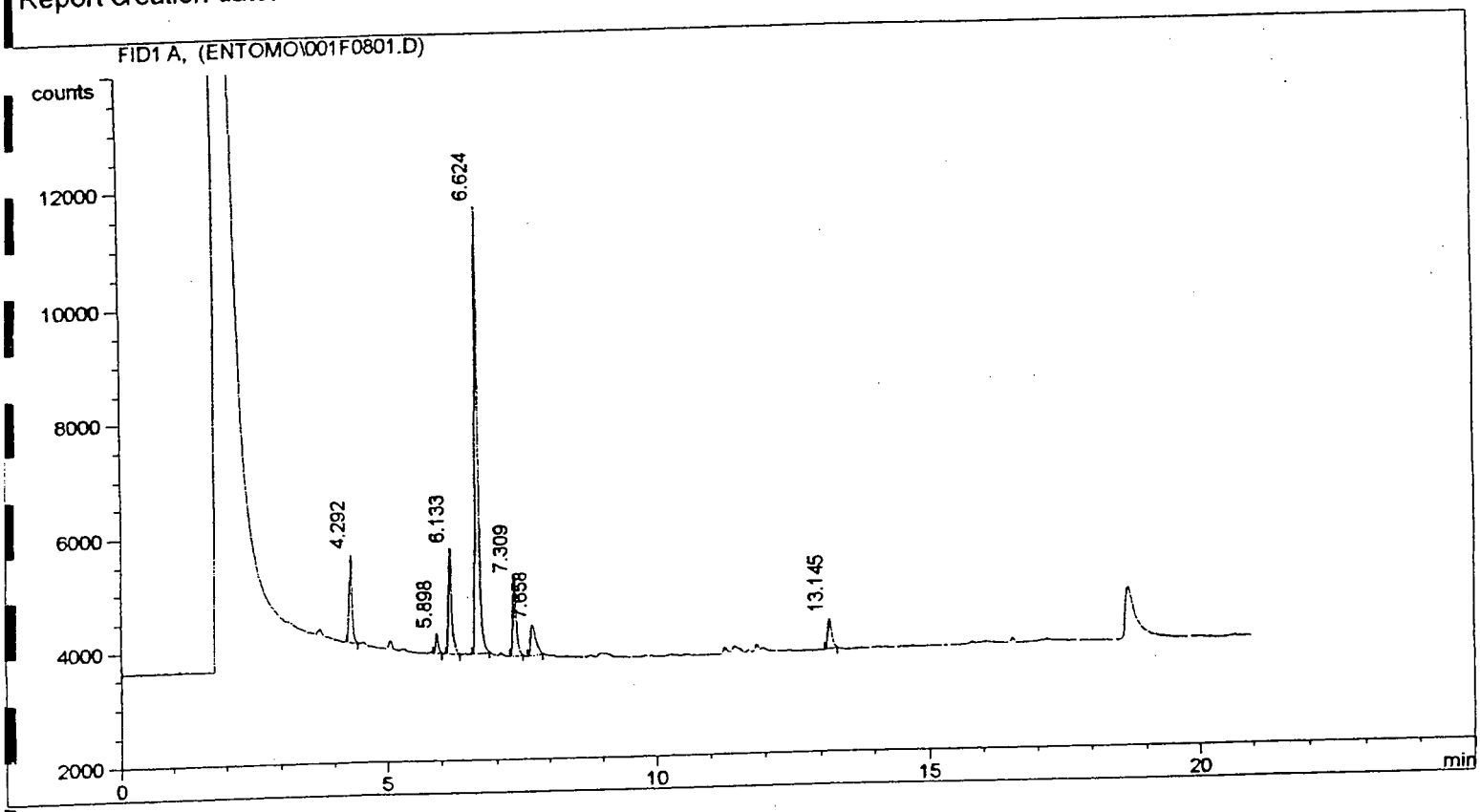
ample Name: BEET3 CONC

Injection Date : Tue, 6. Nov. 2001
Injection time : 5:55:55 PM
Sample Name BEET3 CONC

Seq Line : 8
Vial No. : 1
Inj. No. : 1

Acq Operator : EDUARDO
Acq. Method : FAMELK3.M

Report style : Mark2000
Report creation date: 11/7/01



#	Meas. Ret. T	Peak Type	Height	Area	Area %
1	4.292	PB	1535.495	5667.501	9.842
2	5.898	PP	345.395	1157.401	2.010
3	6.133	PB	1845.289	7325.844	12.722
4	6.624	PB	7783.839	31374.496	54.486
5	7.309	PB	1363.154	5721.583	9.936
6	7.658	PB	539.625	3792.620	6.586
7	13.145	PB	531.537	2542.914	4.416

Instrument : 5890GC

ata file : C:\H CHEM...
ample Name: 85-14A

1

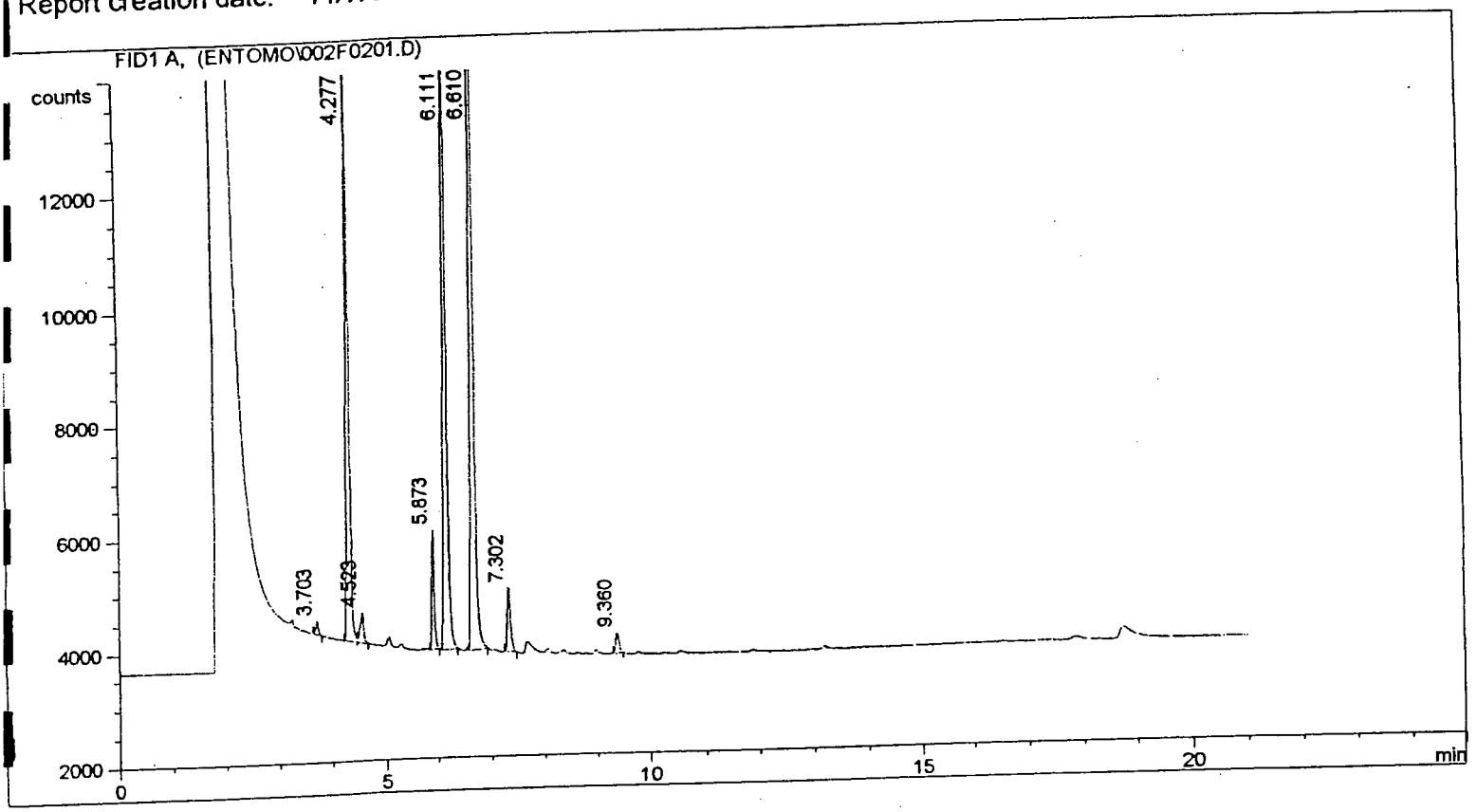
115

Injection Date : Tue, 6. Nov. 2001
Injection time : 3:30:53 PM
Sample Name 85-14A

Seq Line : 2
Vial No. : 2
Inj. No. : 1

Acq Operator : EDUARDO
Acq. Method : FAMELK3.M

Report style : Mark2000
Report creation date: 11/7/01



#	Meas. Ret. T	Peak Type	Height	Area	Area %
1	3.703	BP	237.066	828.868	0.403
2	4.277	PV	10172.760	35813.496	17.421
3	4.523	VB	528.052	2290.071	1.114
4	5.873	PB	2105.169	6731.438	3.274
5	6.111	PB	16492.365	59647.195	29.014
6	6.610	VB	25042.893	94151.125	45.798
7	7.302	BB	1118.774	4613.660	2.244
8	9.360	BB	356.320	1501.665	0.730

Instrument : 5890GC

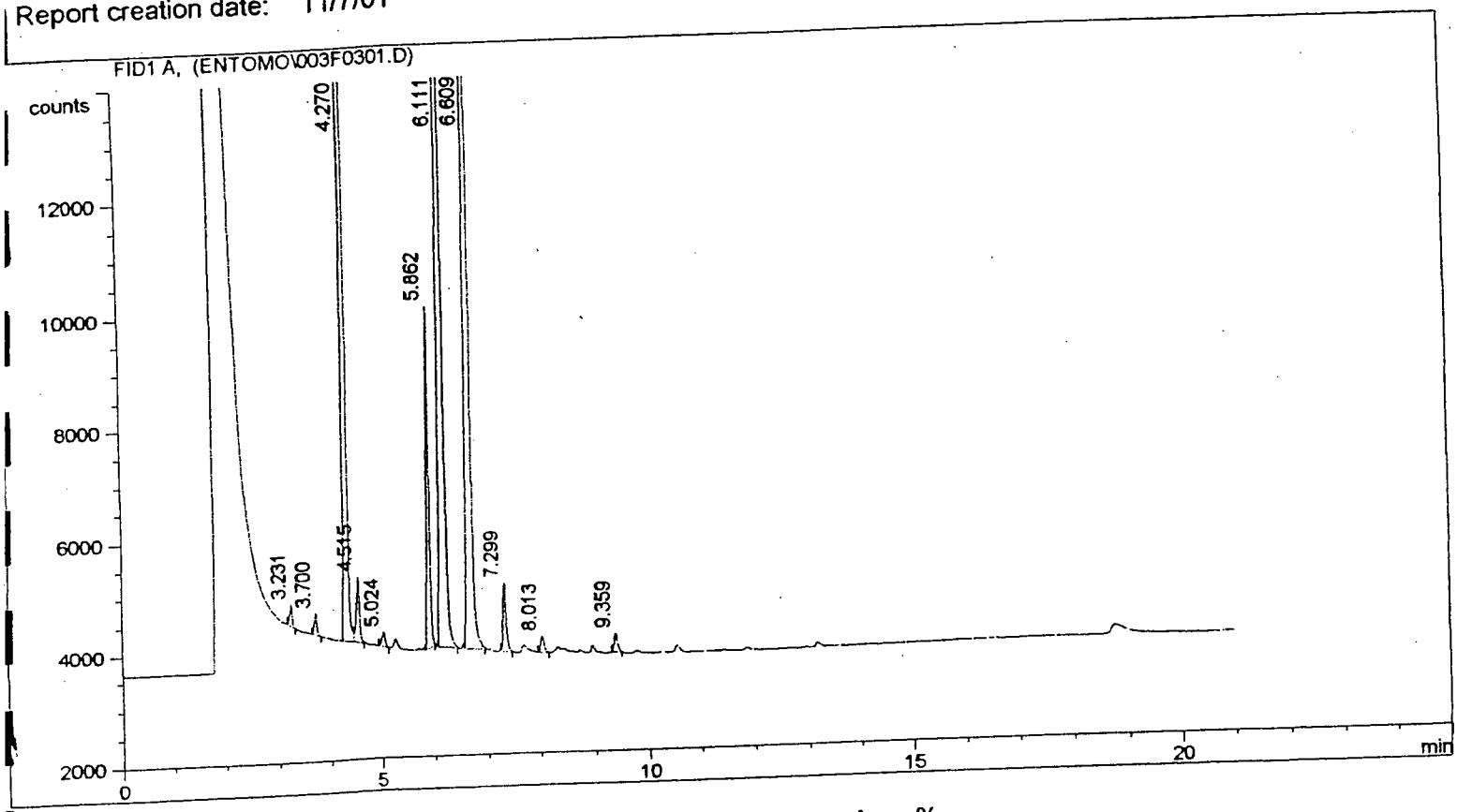
ample Name: 100-1

Injection Date : Tue, 6. Nov. 2001
Injection time : 3:55:02 PM
Sample Name 100-1

Seq Line : 3
Vial No. : 3
Inj. No. : 1

Acq Operator : EDUARDO
Acq. Method : FAMELK3.M

Report style : Mark2000
Report creation date: 11/7/01



#	Meas. Ret. T	Peak Type	Height	Area	Area %
1	3.231	BB	378.486	1203.089	0.253
2	3.700	BB	387.968	1226.685	0.258
3	4.270	PV	29197.275	97583.508	20.522
4	4.515	VB	1172.631	4745.554	0.998
5	5.024	BP	252.597	1068.031	0.225
6	5.862	VB	6105.434	19689.742	4.141
7	6.111	BB	50990.031	177050.922	37.234
8	6.609	VB	46771.109	165361.484	34.776
9	7.299	BB	1213.140	4852.896	1.021
10	8.013	PP	291.508	1313.479	0.276
11	9.359	PB	338.728	1411.078	0.297

Instrument : 5890GC

ata file : C:\HPCHEM\...

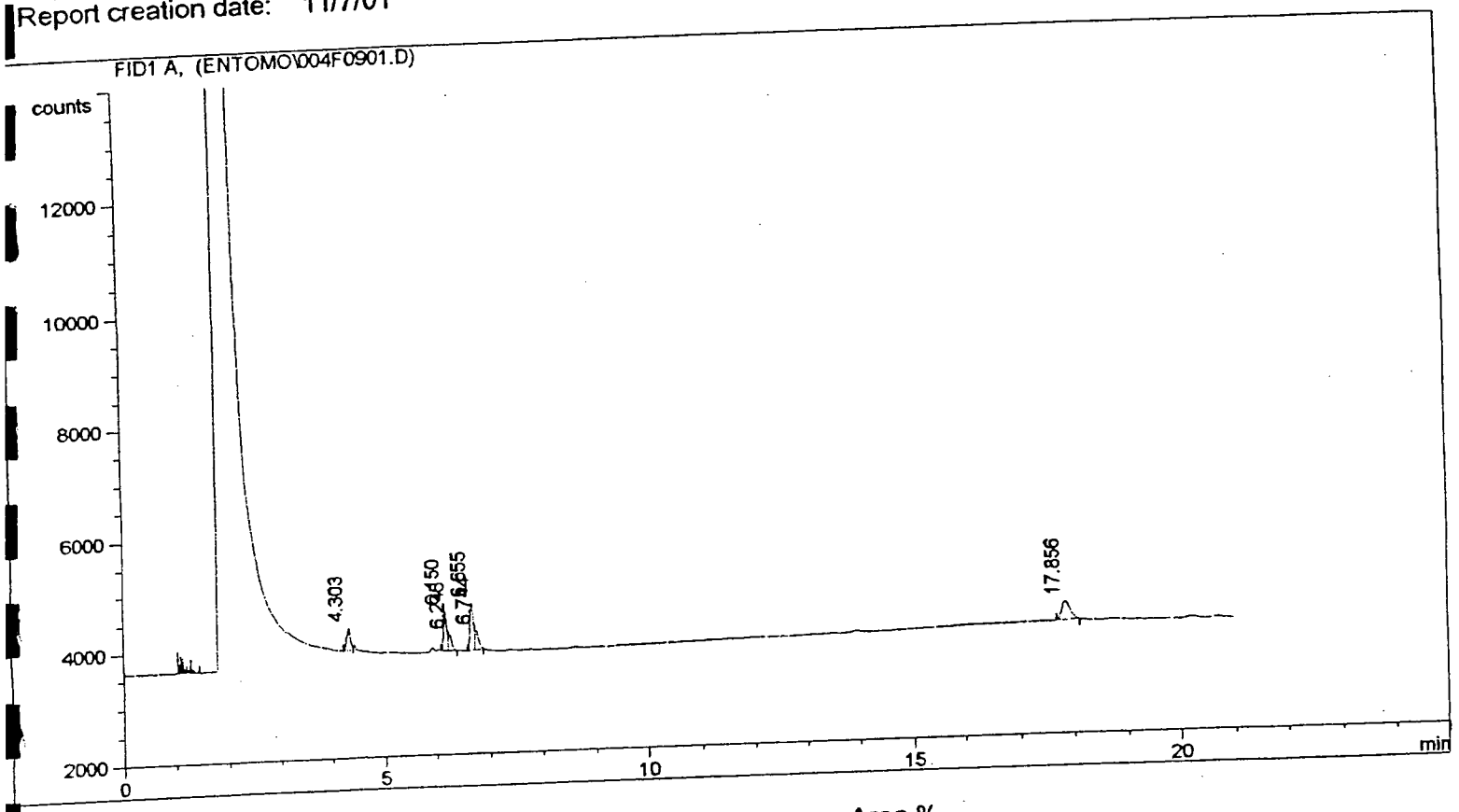
ample Name: 95-2 CONC

Injection Date : Tue, 6. Nov. 2001
Injection time : 6:20:04 PM
Sample Name 95-2 CONC

Seq Line : 9
Vial No. : 4
Inj. No. : 1

Acq Operator : EDUARDO
Acq. Method : FAMELK3.M

Report style : Mark2000
Report creation date: 11/7/01



#	Meas. Ret. T	Peak Type	Height	Area	Area %
1	4.303	PV	407.568	1901.951	12.700
2	6.150	PV	711.079	3091.594	20.644
3	6.245	VB	285.550	1174.281	7.841
4	6.655	PV	850.990	3825.358	25.543
5	6.744	VB	351.170	1462.798	9.768
6	17.856	BB	333.336	3520.015	23.504

Instrument : 5890GC

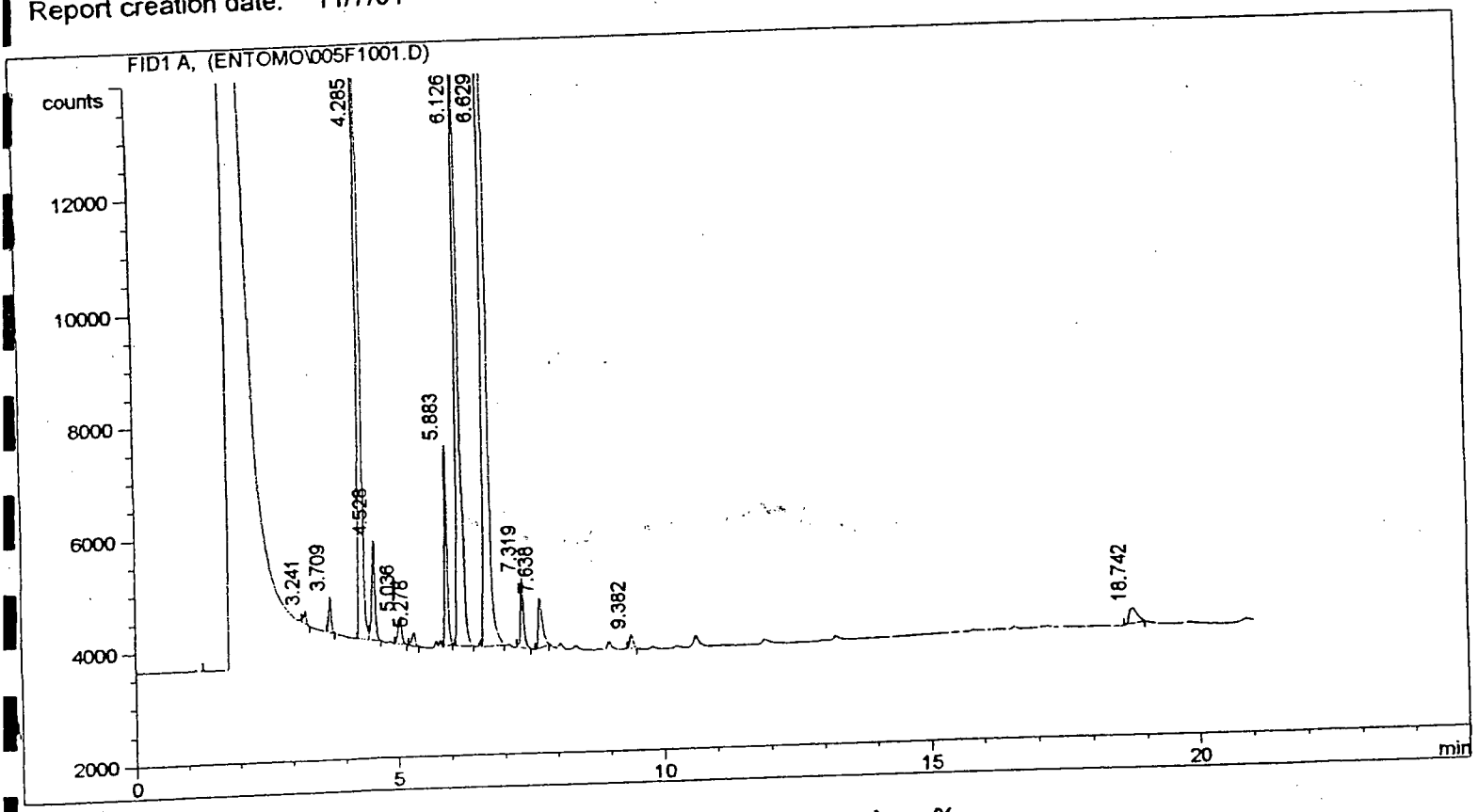
Data file : C:\HPCHEM\2\DATA\ENTOMO\005F1001.D
Sample Name: 01-07 conc 1

Injection Date : Tue, 6. Nov. 2001
Injection time : 6:44:12 PM
Sample Name 01-07 conc

Seq Line : 10
Vial No. : 5
Inj. No. : 1

Acq Operator : EDUARDO
Acq. Method : FAMELK3.M

Report style : Mark2000
Report creation date: 11/7/01



#	Meas. Ret. T	Peak Type	Height	Area	Area %
1	3.241	BP	233.479	697.862	0.202
2	3.709	BB	639.599	2012.796	0.582
3	4.285	BV	20768.074	68988.242	19.959
4	4.528	VB	1758.581	6761.602	1.956
5	5.036	PB	485.157	2205.850	0.638
6	5.278	BP	230.892	968.801	0.280
7	5.883	VB	3572.870	11348.113	3.283
8	6.126	PB	19652.600	70837.477	20.495
9	6.629	VB	45970.555	167852.141	48.563
10	7.319	BB	1239.181	4949.947	1.432
11	7.638	PB	879.950	5064.497	1.465
12	9.382	BB	257.007	1113.157	0.322
13	18.742	PB	283.002	2840.726	0.822

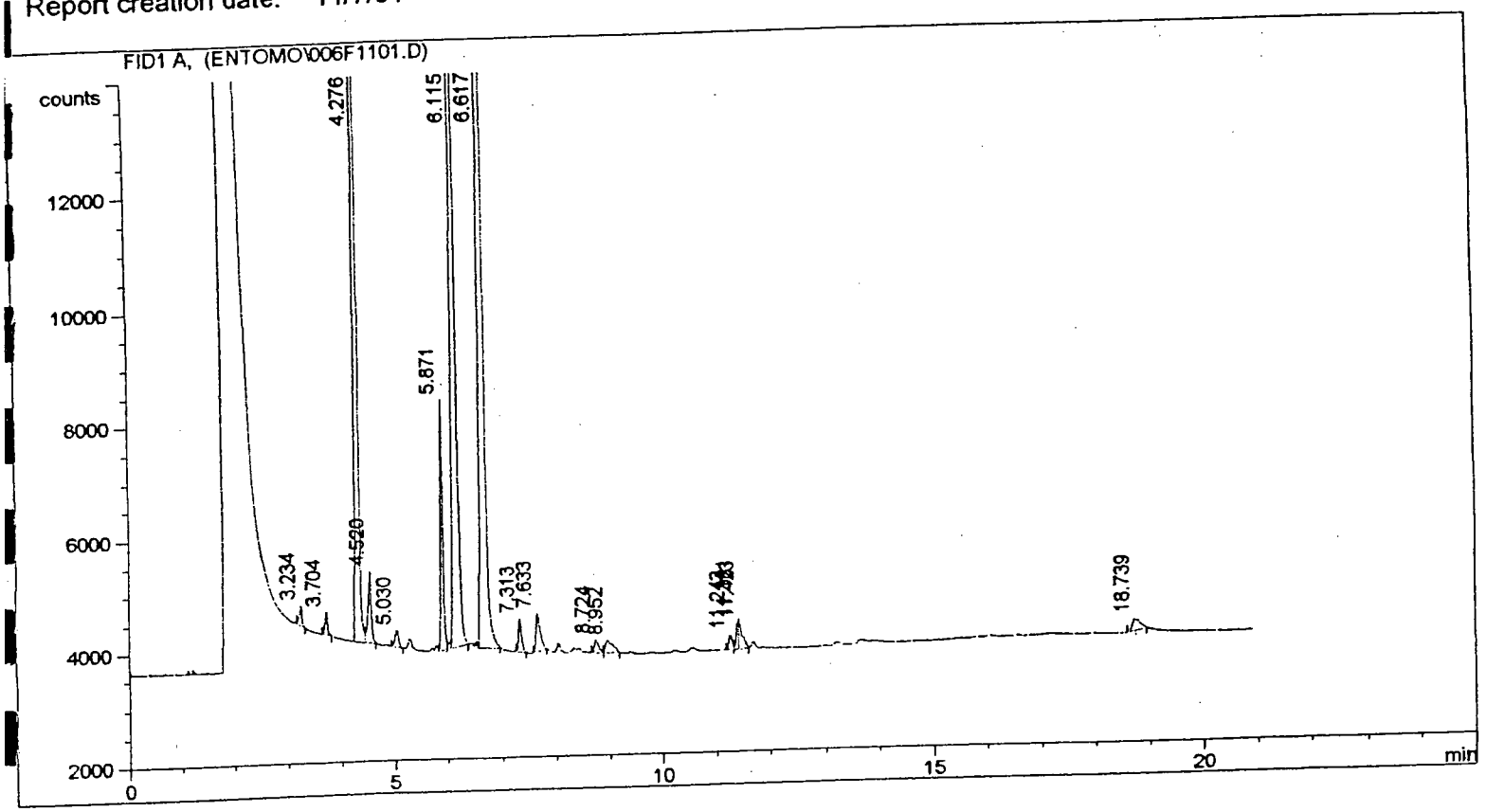
Instrument : 5890GC

Injection Date : Tue, 6. Nov. 2001
Injection time : 7:08:24 PM
Sample Name 24-2B conc

Seq Line : 11
Vial No. : 6
Inj. No. : 1

Acq Operator : EDUARDO
Acq. Method : FAMELK3.M

Report style : Mark2000
Report creation date: 11/7/01



#	Meas. Ret. T	Peak Type	Height	Area	Area %
1	3.234	BB	353.486	1128.001	0.269
2	3.704	BB	409.500	1288.838	0.307
3	4.276	PV	22699.533	74634.070	17.767
4	4.520	VB	1260.879	5178.262	1.233
5	5.030	PB	297.742	1296.982	0.309
6	5.871	VV	4429.290	13966.947	3.325
7	6.115	VP	36771.512	131049.211	31.197
8	6.617	VB	49954.148	174673.047	41.583
9	7.313	BB	589.242	2310.101	0.550
10	7.633	PB	680.211	3900.904	0.929
11	8.724	PB	238.488	1250.128	0.298
12	8.952	BB	218.174	2085.159	0.496
13	11.243	PV	275.208	1123.337	0.267
14	11.381	VV	476.592	1678.311	0.400
15	11.423	VB	448.144	2319.244	0.552
16	18.739	PB	220.192	2181.289	0.519

Instrument : 5890GC

Data file : C:\HP CHEM\...

1

Sample Name: 73-21

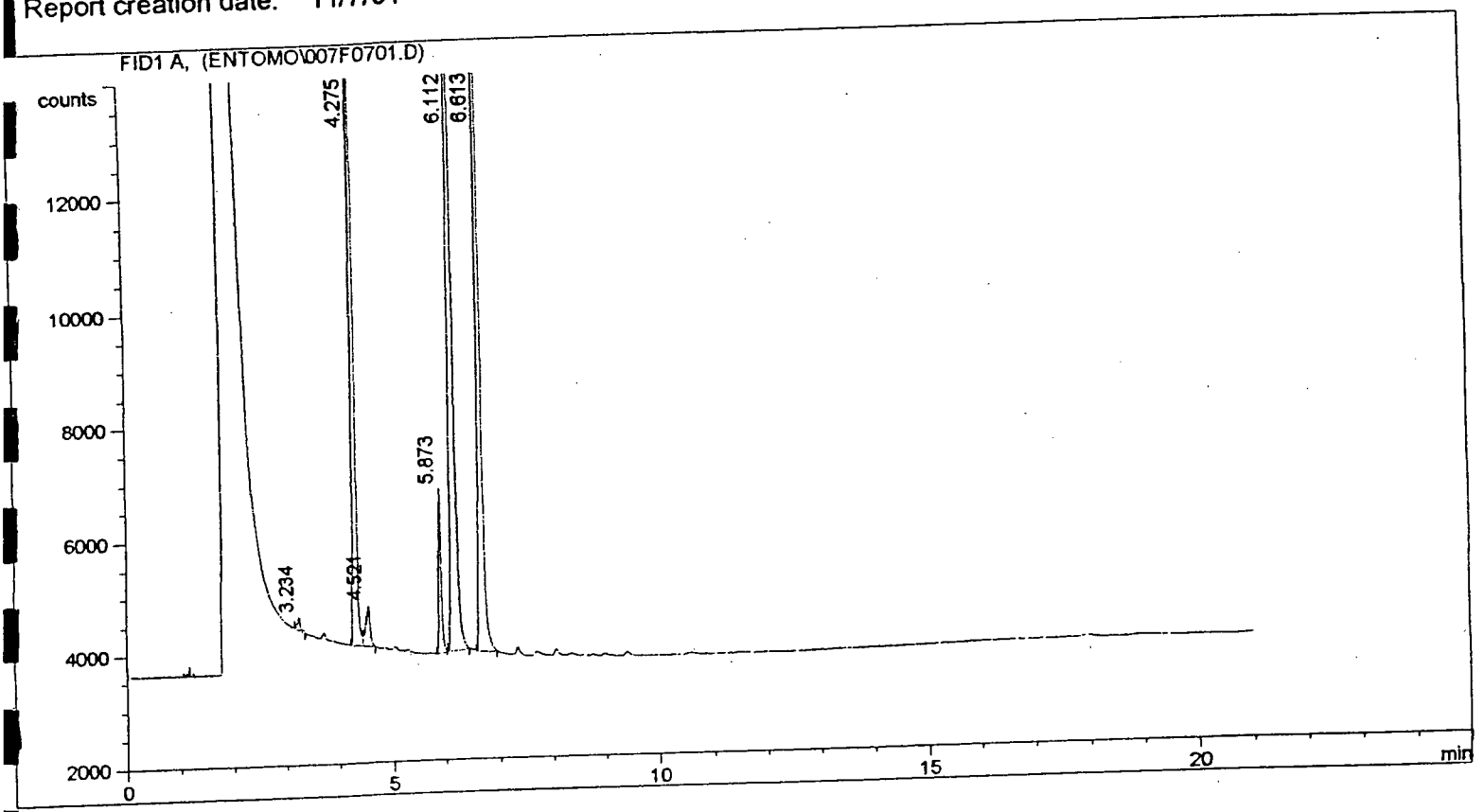
Injection Date : Tue, 6. Nov. 2001
Injection time : 5:31:43 PM
Sample Name 73-21

Seq Line : 7
Vial No. : 7
Inj. No. : 1

120

Acq Operator : EDUARDO
Acq. Method : FAMELK3.M

Report style : Mark2000
Report creation date: 11/7/01



#	Meas. Ret. T	Peak Type	Height	Area	Area %
1	3.234	BB	216.494	850.762	0.338
2	4.275	PB	16010.773	56787.437	22.591
3	4.521	BB	715.577	3358.077	1.336
4	5.873	PB	2896.896	9727.482	3.870
5	6.112	BB	29328.527	108461.594	43.149
6	6.613	VB	18661.885	72181.953	28.716

Instrument : 5890GC

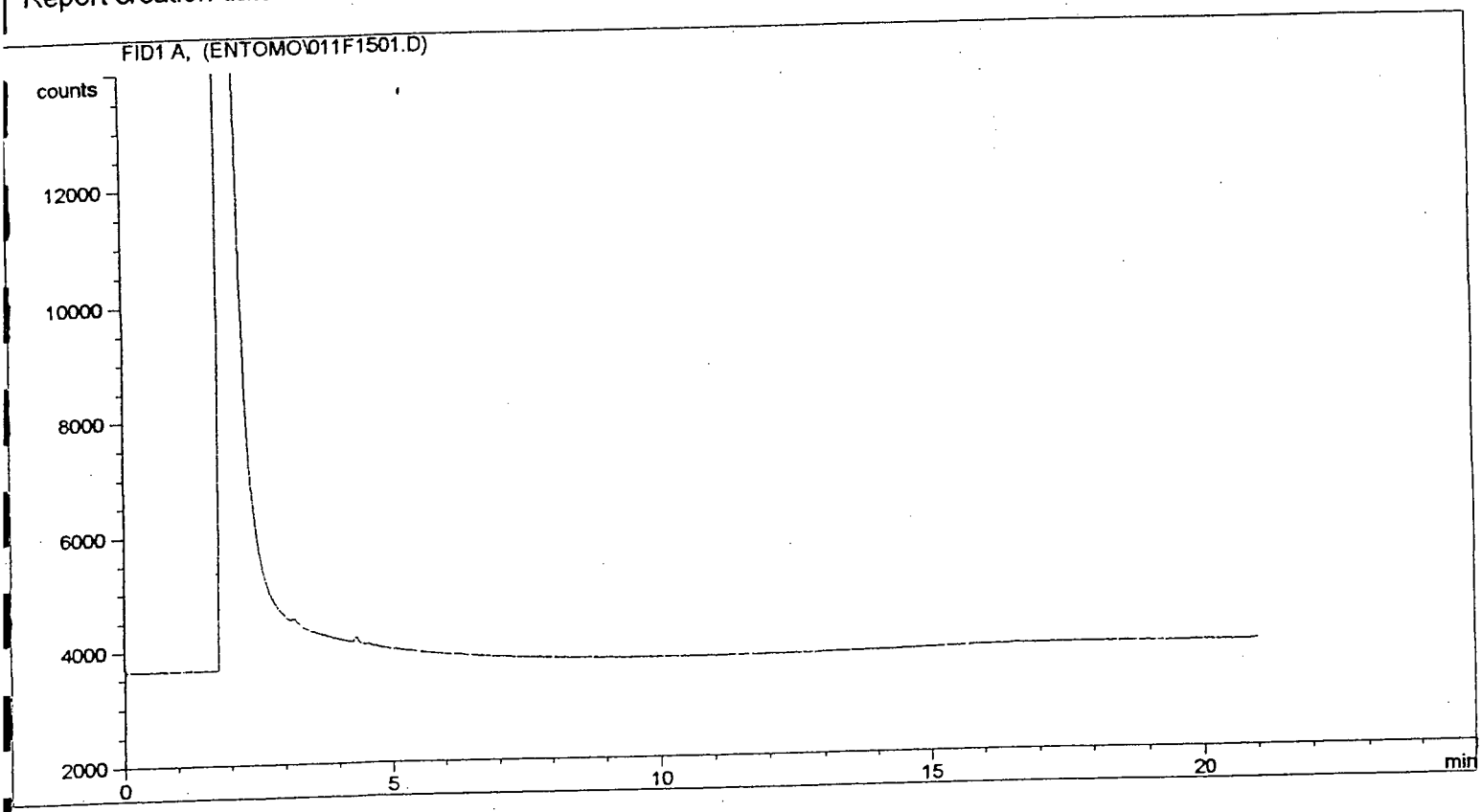
Data file : C:\HPCHEM\2\DATA\ENTOMO\011F1501.D
Sample Name: BLANK 1

Injection Date : Tue, 6. Nov. 2001
Injection time : 8:44:56 PM
Sample Name BLANK

Seq Line : 15
Vial No. : 11
Inj. No. : 1

Acq Operator : EDUARDO
Acq. Method : FAMELK3.M

Report style : Mark2000
Report creation date: 11/7/01



#	Meas. Ret. T	Peak Type	Height	Area	Area %

Instrument : 5890GC

ata file : C:\HPCHEM\2\DATA\ENTOMO\...
ample Name: STDS

1

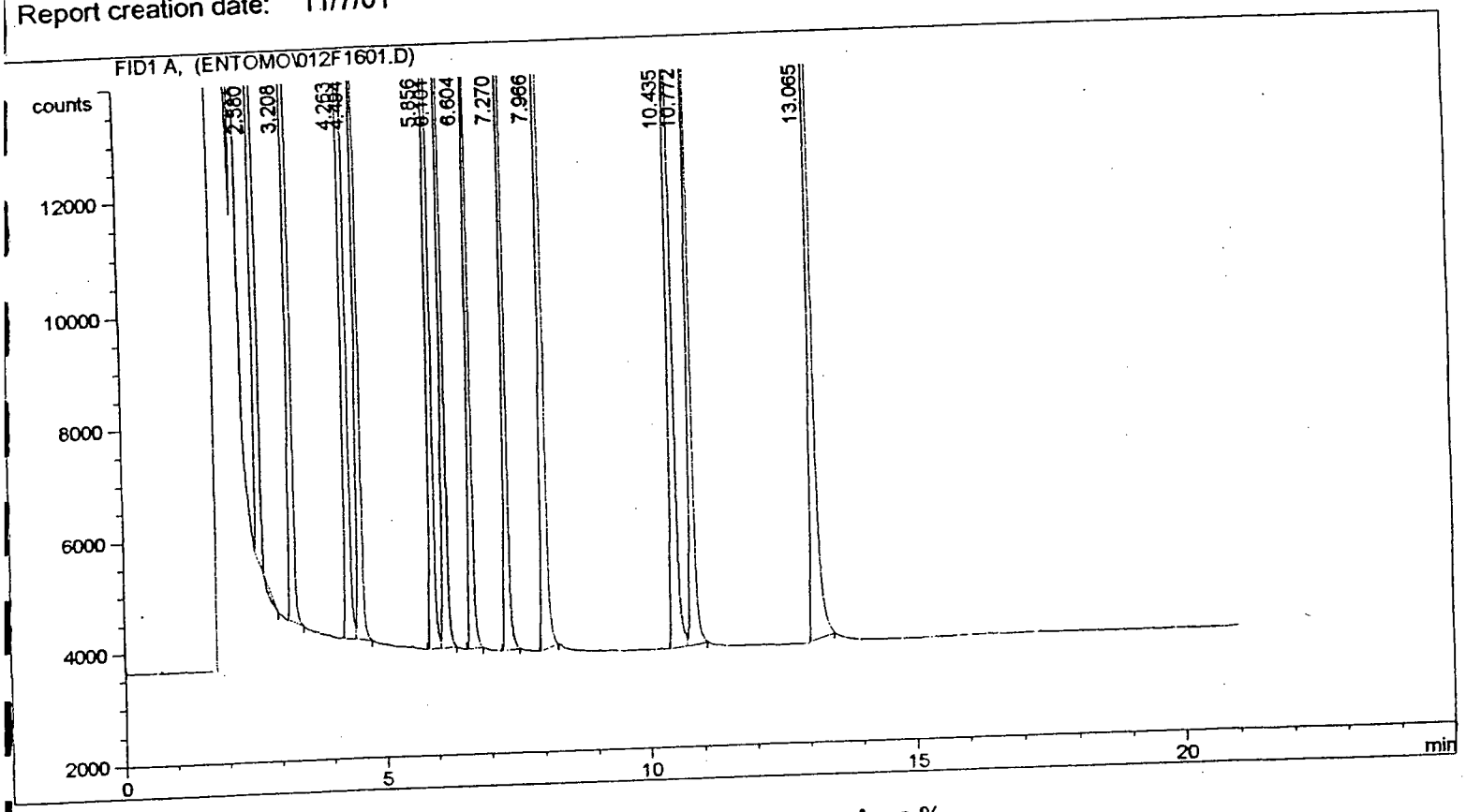
Injection Date : Tue, 6. Nov. 2001
Injection time : 9:09:04 PM
Sample Name STDS

Seq Line : 16
Vial No. : 12
Inj. No. : 1

122

Acq Operator : EDUARDO
Acq. Method : FAMELK3.M

Report style : Mark2000
Report creation date: 11/7/01



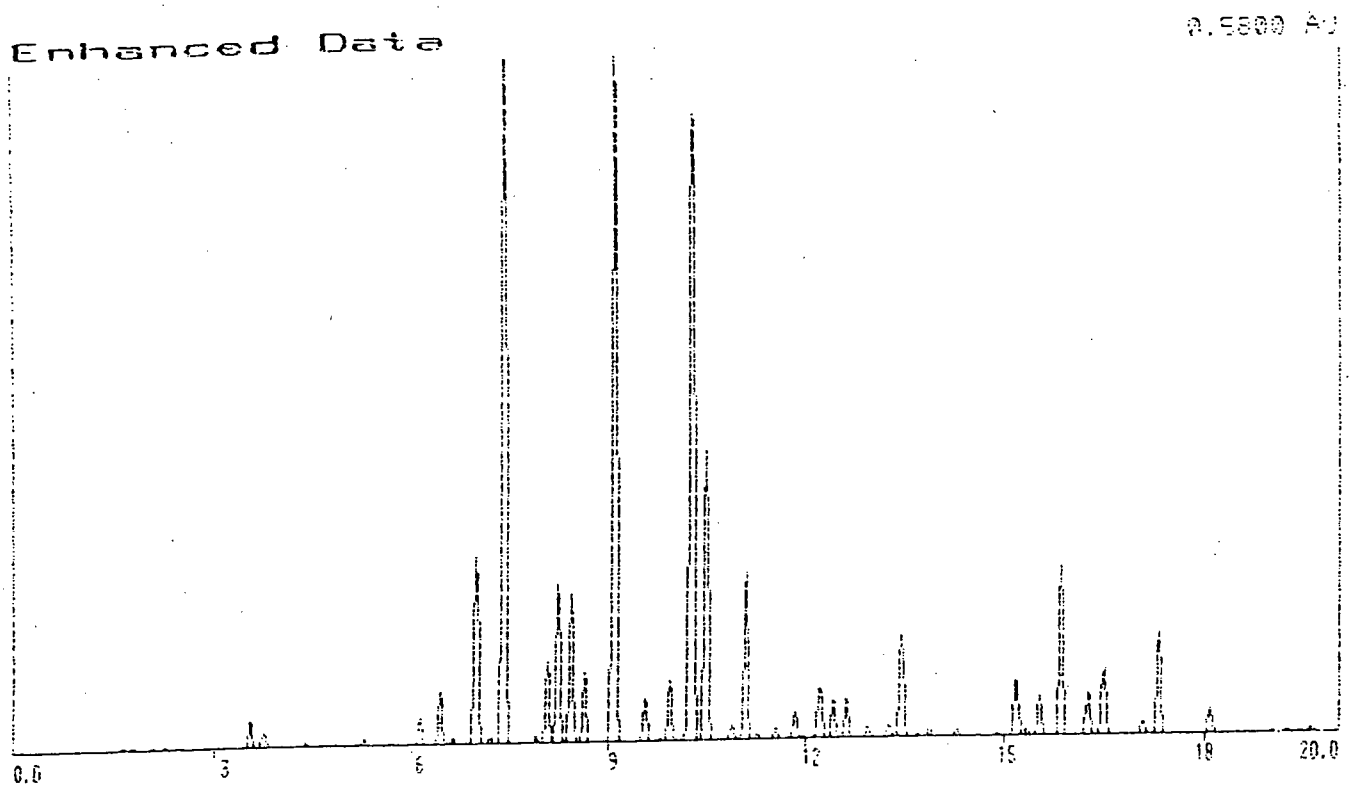
#	Meas. Ret. T	Peak Type	Height	Area	Area %	
1	2.580	BP	38450.629	120311.766	9.917	— 12:0
2	3.208	PB	38380.203	121883.875	10.046	— 14:0
3	4.263	BV	49080.367	162526.078	13.396	— 16:0
4	4.494	VB	20792.988	73143.531	6.029	— 16:1 (Δ9)
5	5.856	PV	33984.629	116429.859	9.597	— 18:0
6	6.101	VB	20751.988	72723.008	5.994	— 18:1 (Δ9)
7	6.604	PB	21109.391	72149.984	5.947	— 18:2 (Δ9, Δ12)
8	7.270	PB	18317.959	67218.906	5.540	— 18:3 (Δ9, Δ12, Δ15)
9	7.966	PB	32989.437	120433.547	9.927	— 20:0
10	10.435	PV	29639.160	120315.695	9.917	— 22:0
11	10.772	VB	18022.639	67408.570	5.556	— 22:1 (Δ11)
12	13.065	PB	20405.035	98683.641	8.134	— 24:0

Instrument : 5890GC

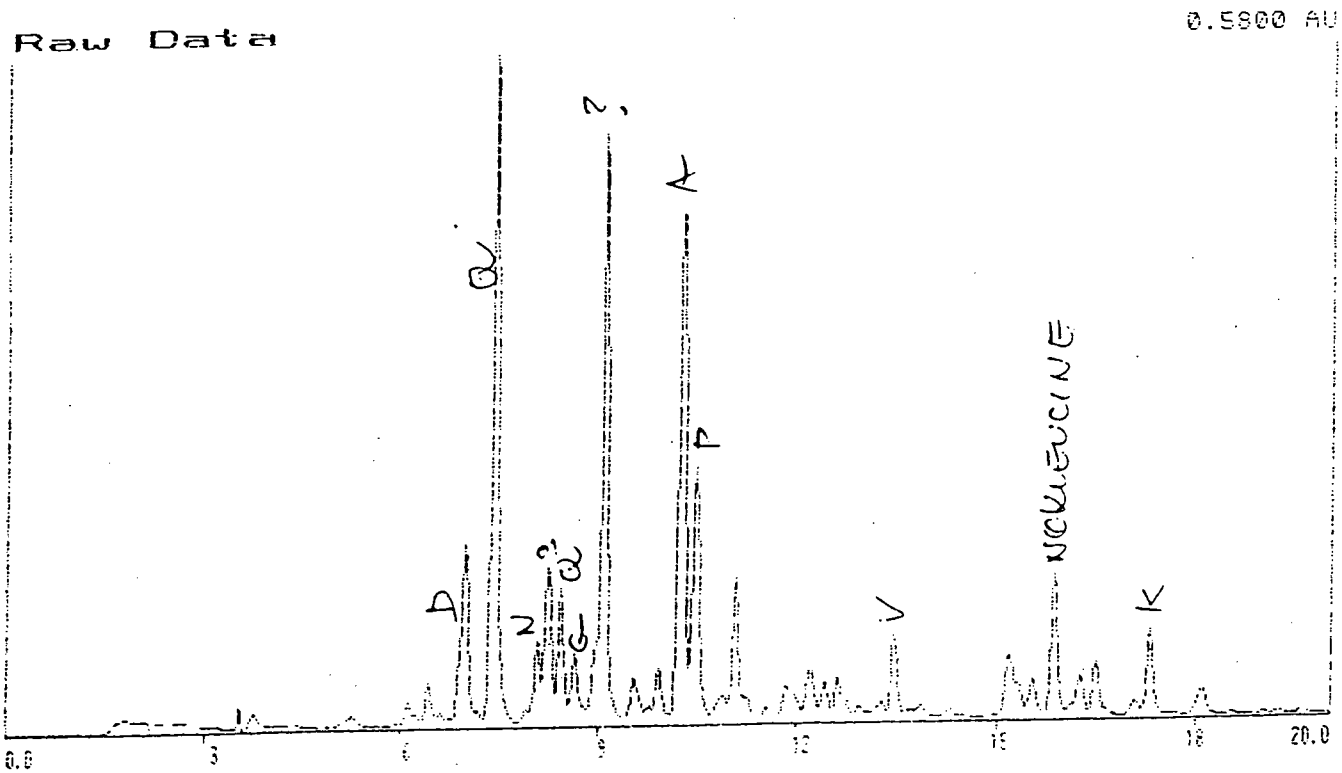
Figure B.3 Chromatograms of Amino acid analysis from sporocarps of *Cordyceps cyanensis*. Included are: Chromatogram Report, Mol Percent Report, and Typical Amino Acid Analysis Results (Hydrolysis Test Peptide).

Sample ID: 14501009 Initiated 5/25/99 10:17am BASELINE CORRECTED

Enhanced Data



Raw Data



Sample ID: 14501009 (initiated 5/25/99 10:17pm) BASELINE CORRECTED

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Data Start : 0.00 min
Data Duration : 20.00 min
Peak Ht Threshold : 999 uAU

Sampling Interval: 1.0 sec
Samples In Run : 72
Operator ID : P
Int. Std. Amt : 500 pmol

Calibration File : 2B-05CAL (initiated 5/20/99 9:45am)
Reference Time : 0.00 min
Reference Offset 1: 0.00 min
Reference Offset 2: 0.00 min
ISTD Peak ID : NOR

Integration Interval: 8.0 to 19.0 min

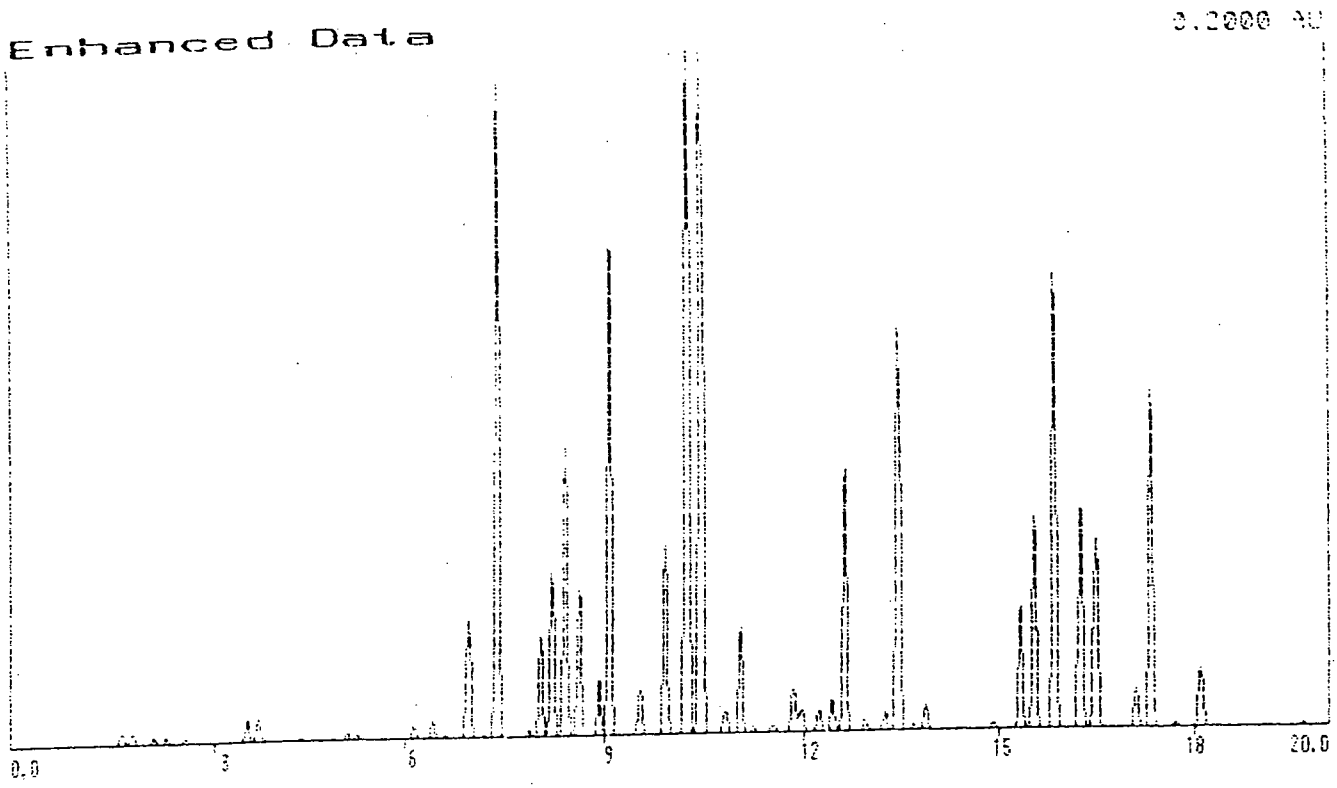
PEAK ID	RET. TIME min	PMOL BY HEIGHT	PMOL correc. INT STD	MOL %
Aspartic Acid	6.97	951.71	954.62	5.09
Glutamic Acid	7.37	4665.63	4188.65	24.94
Serine	8.22	1129.97	1014.69	5.04
Glycine	8.65	489.73	439.77	2.62
Histidine	9.08	4511.88	4051.59	24.12
Arginine	9.59	214.79	192.88	1.15
Threonine	9.93	380.72	341.88	2.04
Alanine	10.23	3335.98	2995.65	17.83
Proline	10.45	1489.41	1337.46	7.98
Tyrosine	12.63	165.87	148.95	0.89
Valine	13.45	484.35	434.94	2.59
Methionine	13.70	20.00	17.96	0.11
Cysteine	14.30	46.15	41.44	0.25
Isoleucine	15.35	43.98	39.50	0.24
Leucine	15.55	209.45	188.08	1.12
NOR	15.85	1113.81	1000.00	ISTD
Phenylalanine	16.27	226.54	203.43	1.21
Lysine	17.32	340.74	305.98	1.82

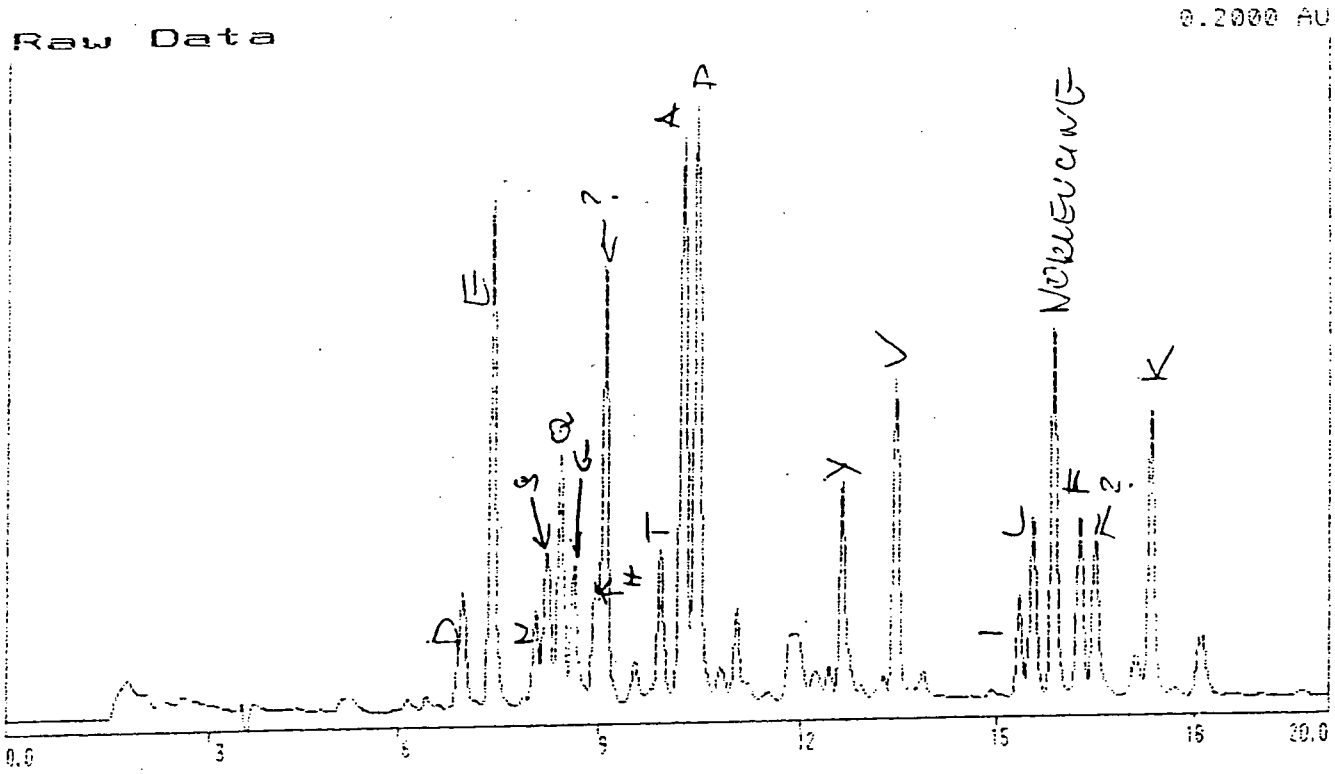
TOTAL PMOLS RECOVERED 16798.47

Minimum Peak Threshold: 999 uAU (4 peaks below threshold)
(44 peaks found)
(18 peaks matched)

12 µl Dilf 1

Sample ID: 14501025 Initiated 5/29/98 7:01am BASELINE CORRECTED





Sample ID: 14501004 Initiated 5/25/89 6:13pm/ BASELINE CORRECTED

Turntable Position: 2 A
Data Start : 0.00 min
Data Duration : 20.00 min
Peak Ht Threshold : 339 uAU

Sampling Interval: 1.0 sec
Samples In Run : 72
Operator ID : 4
Int. Std. Amt : 1000 pmol

Calibration File : 25-05DAL
Reference Time : 0.00 min
Reference Offset 1: 0.00 min
Reference Offset 2: 0.00 min

(initiated 5/26/89 9:35am)
ISTD Peak ID : NOR

Integration Interval: 6.0 to 19.0 min

PEAK ID	RET. TIME min	CAL. TIME min	PEAK HEIGHT uAU	PMOL BY HEIGHT	PMOL correc. INT STD
	6.18	-----	4065	----------	----------
	6.47	-----	5439	----------	----------
Aspartic Acid	7.00	6.98	35920	212.42	203.65
	7.20	-----	1238	----------	----------
Glutamic Acid	7.40	7.40	196985	1257.08	1205.17
	7.68	-----	2556	----------	----------
	8.07	-----	31889	----------	----------
Serine	8.23	8.22	52722	432.53	414.67
	8.45	-----	92240	----------	----------
Glycine	8.67	8.65	49369	399.17	382.68
Histidine	8.97	8.97	19037	141.02	136.18
	9.12	-----	146072	----------	----------
Arginine	9.55	9.55	15241	90.80	87.05
Threonine	9.95	9.93	65141	470.98	451.44
Alanine	10.27	10.27	215164	1345.13	1289.58
Proline	10.47	10.47	217093	1298.25	1233.13
	10.83	-----	7847	----------	----------
	11.08	-----	33012	----------	----------
	11.28	-----	2041	----------	----------
	11.57	-----	3085	----------	----------
	11.87	-----	12781	----------	----------
	12.00	-----	7037	----------	----------
	12.25	-----	6534	----------	----------
	12.48	-----	10661	----------	----------
Tyrosine	12.65	12.63	79800	400.89	384.34
	12.87	-----	3024	----------	----------
	13.28	-----	5986	----------	----------
Valine	13.48	13.47	121335	651.52	624.20
Methionine	13.73	13.77	2257	14.23	13.64
	13.90	-----	8054	----------	----------
	14.95	-----	2626	----------	----------
Isoleucine	15.37	15.35	37906	228.20	218.77
Leucine	15.57	15.57	64118	408.78	392.84
NOR	15.88	15.89	138434	1043.07	1000.00
Phenylalanine	16.28	16.28	66492	418.77	401.48
	16.53	-----	57723	----------	----------
	17.12	-----	11953	----------	----------
Lysine	17.35	17.35	104262	405.45	388.71
	17.73	-----	1174	----------	----------
	18.12	-----	18237	----------	----------

Typical Amino Acid Analysis Results - Hydrolysis Test Peptide

Amino acid sequence: mol wt = 2153

H-Cys-Pro-Asp-Phe-Gly-His-Ile-Ala-Met-Glu-Leu-Ser-Val-Arg-Thr-Trp-Lys-Tyr-OH

EXPECTED RECOVERIES

Amino Acid	Range
Ala	1.0*
Arg	0.85 -1.0
Asp	0.8 -1.0
Glu	0.8 -0.95
Gly	1.05 -1.2
His	0.75 -0.9
Ile	0.75 -0.9
Leu	0.9 -1.05
Lys	0.85 -1.05
Met	0.65 -0.85
Phe	0.85 -1.0
Pro	0.9 -1.1
Ser	0.7 -0.9
Thr	0.85 -0.95
Tyr	0.75 -0.9
Val	0.9 -1.02

* Recoveries are normalized to Ala = 1

Amount hydrolyzed = 0.5µg

Cys and Trp are not included because they are mostly destroyed during acid hydrolysis

Standard hydrolyzer cycle, 6N HCl, no scavengers/reductants

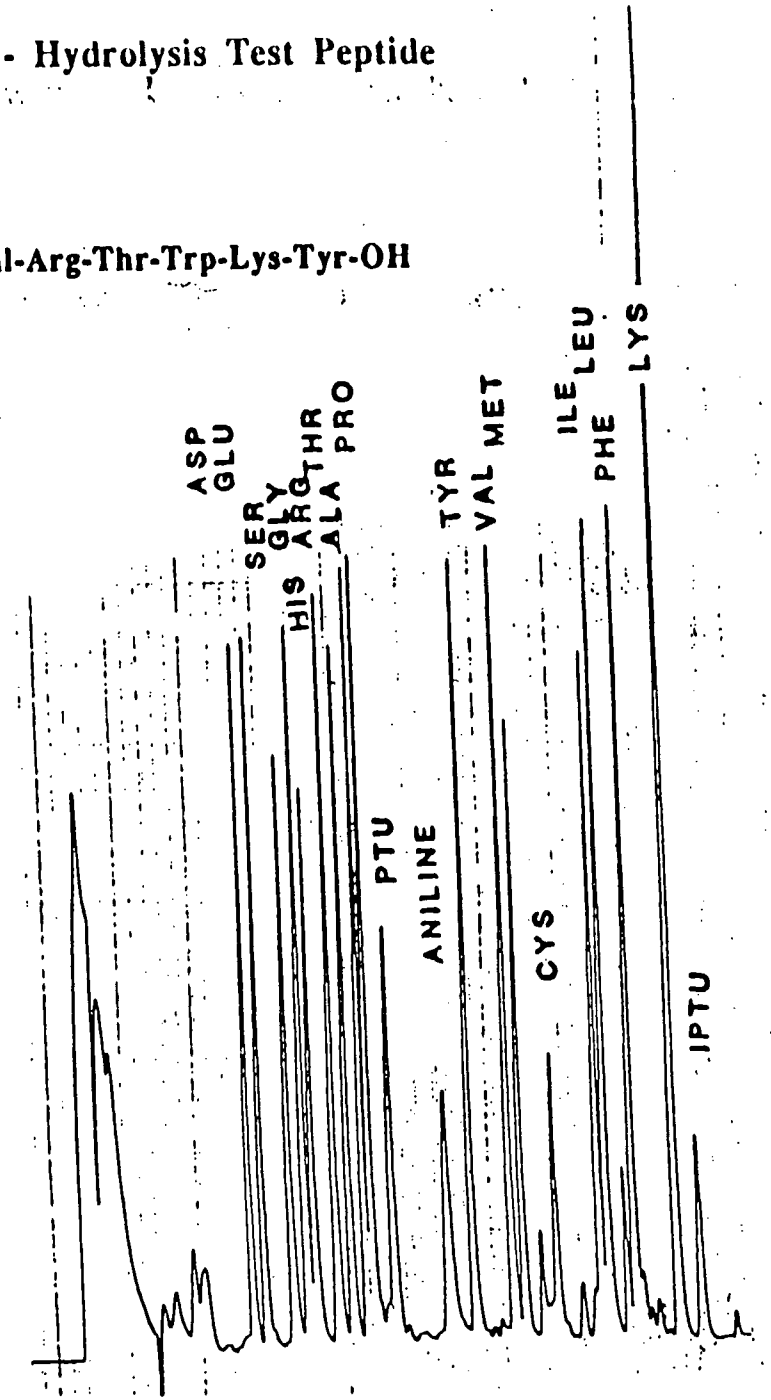
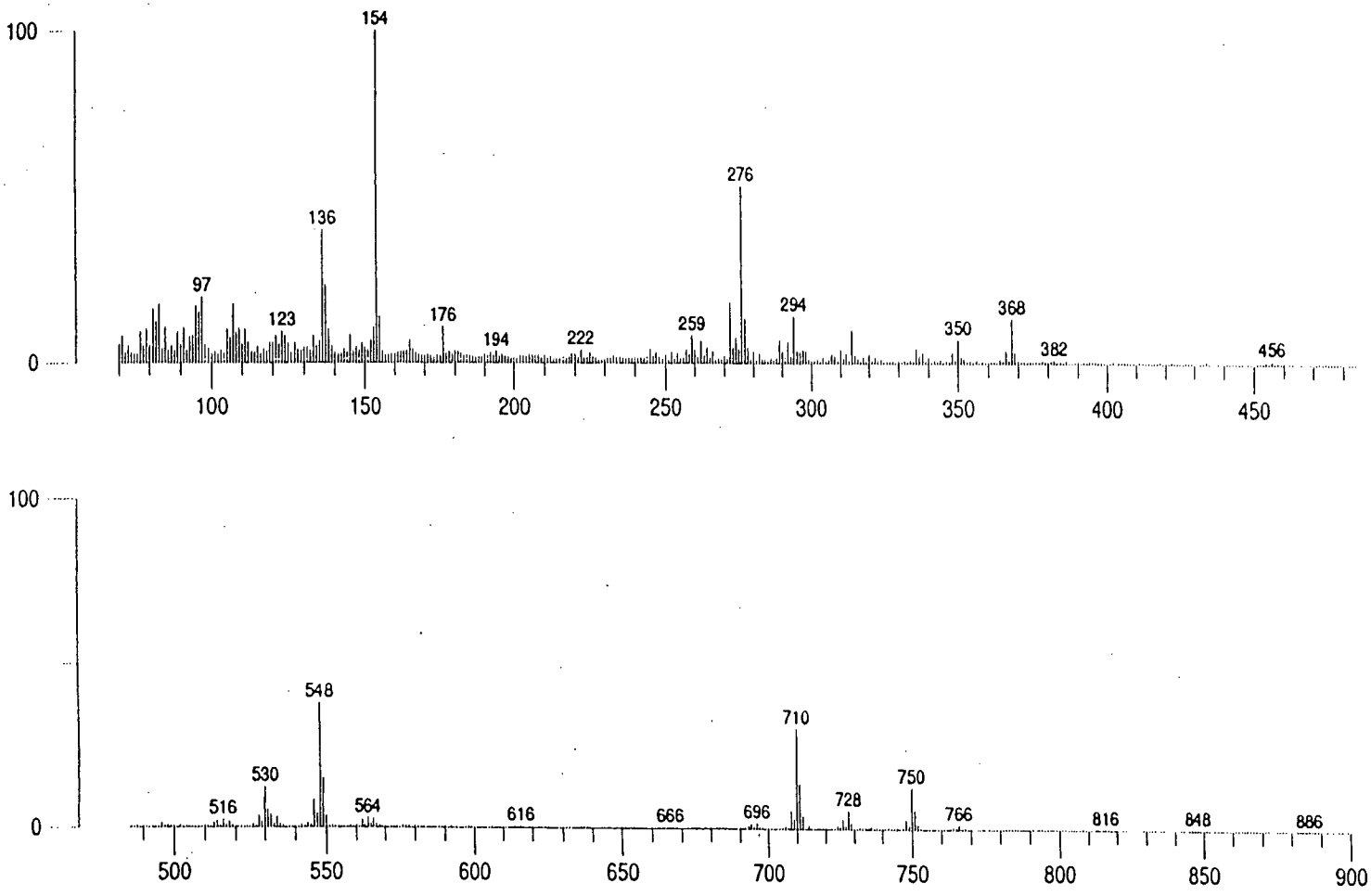


Figure B.4 Physicochemical data used to determine the structure of the cerebroside (4E,8E)-N-2-hydroxyhexadecanoyl-1-O-β-glucanopyranosyl-9-methyl-C^{18}-sphinga-4,8-diene. Included are: a) low resolution FAB Mass Spectrum, b) ^{13}C-NMR Spectrum, c) ^{1}H-^{1}H COSY Spectrum, d) ^{1}H-NMR Spectrum.

E Jewel

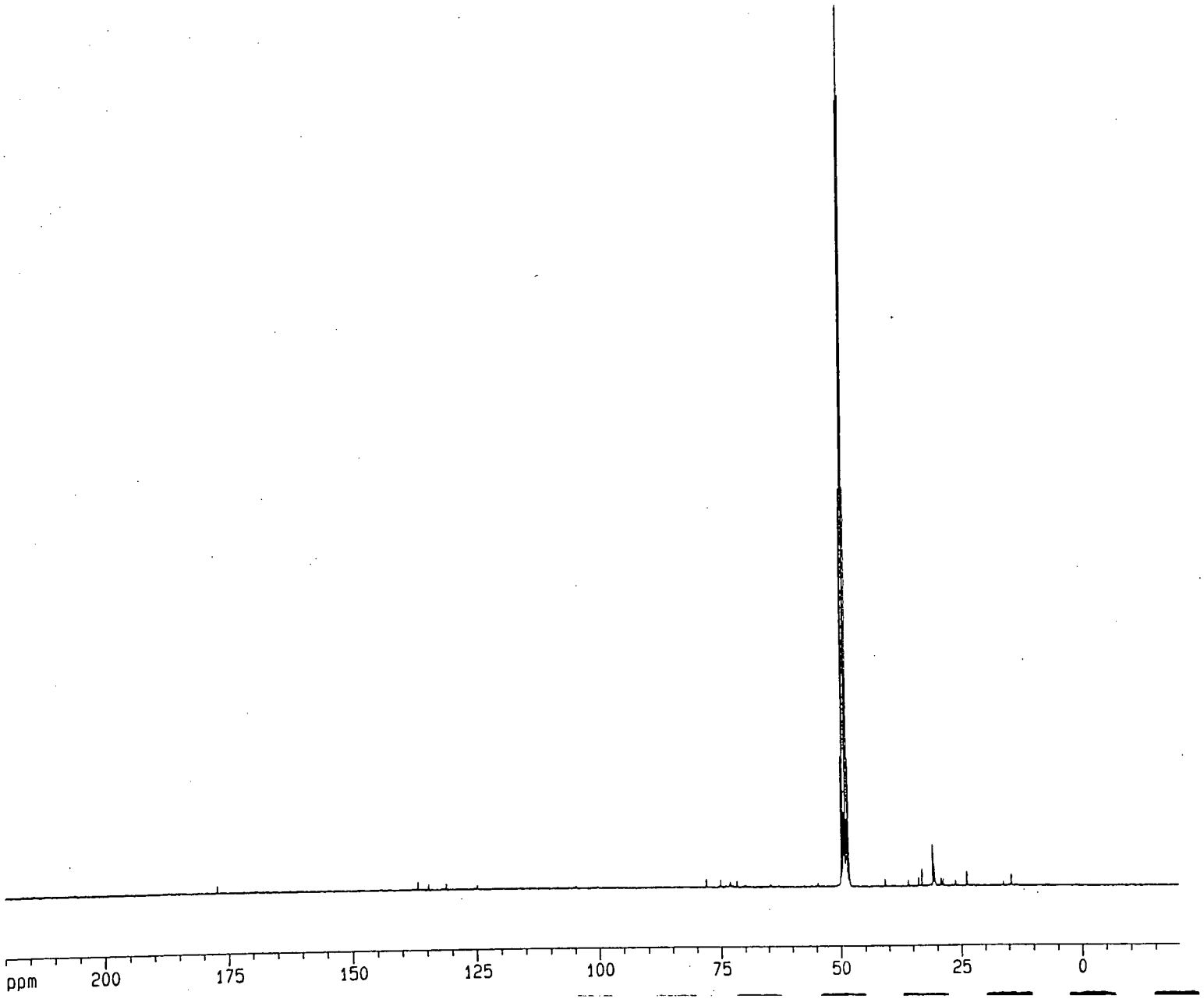
Ed-14

lsims14358 Scan 1 (Av 2-6 Acq) 100%=6877 mv 16 May 91 13:29
LRP +LSIMS SL11362: Ed-14 * Matrix: 3-NBA



sample no: 6002 e.jovel ed-14 /mta

13C bb cd3od



Current Data Parameters

NAME	jovel6002
EXPNO	2
PROCNO	1

F2 - Acquisition Parameters

Date_	20010524
Time	4.48
INSTRUM	av300
PROBHD	5 mm QNP 1H/
PULPROG	zgpg30
TD	65536
SOLVENT	Aceton
NS	12288
DS	4
SWH	18832.393 Hz
FIDRES	0.287360 Hz
AQ	1.7400308 sec
RG	16384
DW	26.550 usec
DE	37.93 usec
TE	300.0 K
D1	2.00000000 sec
d11	0.03000000 sec
d12	0.00002000 sec

======== CHANNEL f1 ========

NUC1	13C
P1	6.50 usec
PL1	0.00 dB
SFO1	75.4755190 MHz

======== CHANNEL f2 ========

CPDPRG2	waltz16
NUC2	1H
PCPD2	70.00 usec
PL2	120.00 dB
PL12	23.60 dB
PL13	28.60 dB
SFO2	300.1312005 MHz

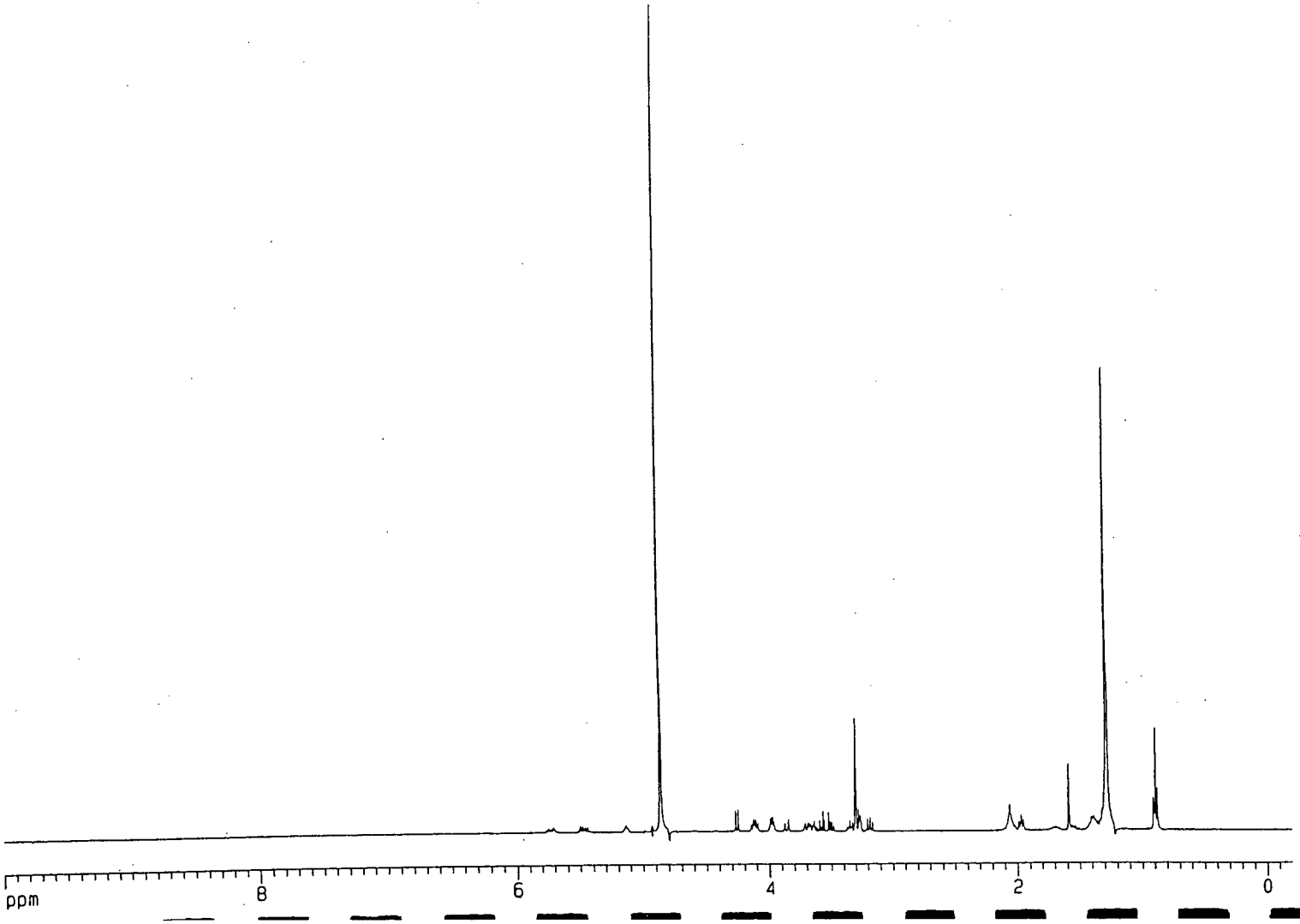
F2 - Processing parameters

SI	32768
SF	75.4676423 MHz
WDW	EM
SSB	0
LB	2.00 Hz
GB	0
PC	2.00

1D NMR plot parameters

CX	20.00 cm
CY	15.00 cm
F1P	220.000 ppm
F1	16602.88 Hz
F2P	-20.000 ppm
F2	-1509.35 Hz
PPMCM	12.00000 ppm/cm
HZCM	905.61176 Hz/cm

JOB NO: 6133 D MING Ed-14
dm6133 1 1



Current Data Parameters

NAME	dm6133
EXPNO	1
PROCNO	1

F2 - Acquisition Parameter

Date_	20010606
Time	9.41
INSTRUM	av400
PROBHD	5 mm BBI 1H-
PULPROG	zg30
TD	32768
SOLVENT	MeOH
NS	32
DS	2
SWH	4990.020 Hz
FIDRES	0.152283 Hz
AQ	3.2834036 se
RG	90.5
DW	100.200 us
DE	6.00 us
TE	300.0 K
D1	1.00000000 se

======== CHANNEL f1 ======

NUC1	1H
P1	10.00 us
PL1	4.30 dB
SFO1	400.1320000 MH

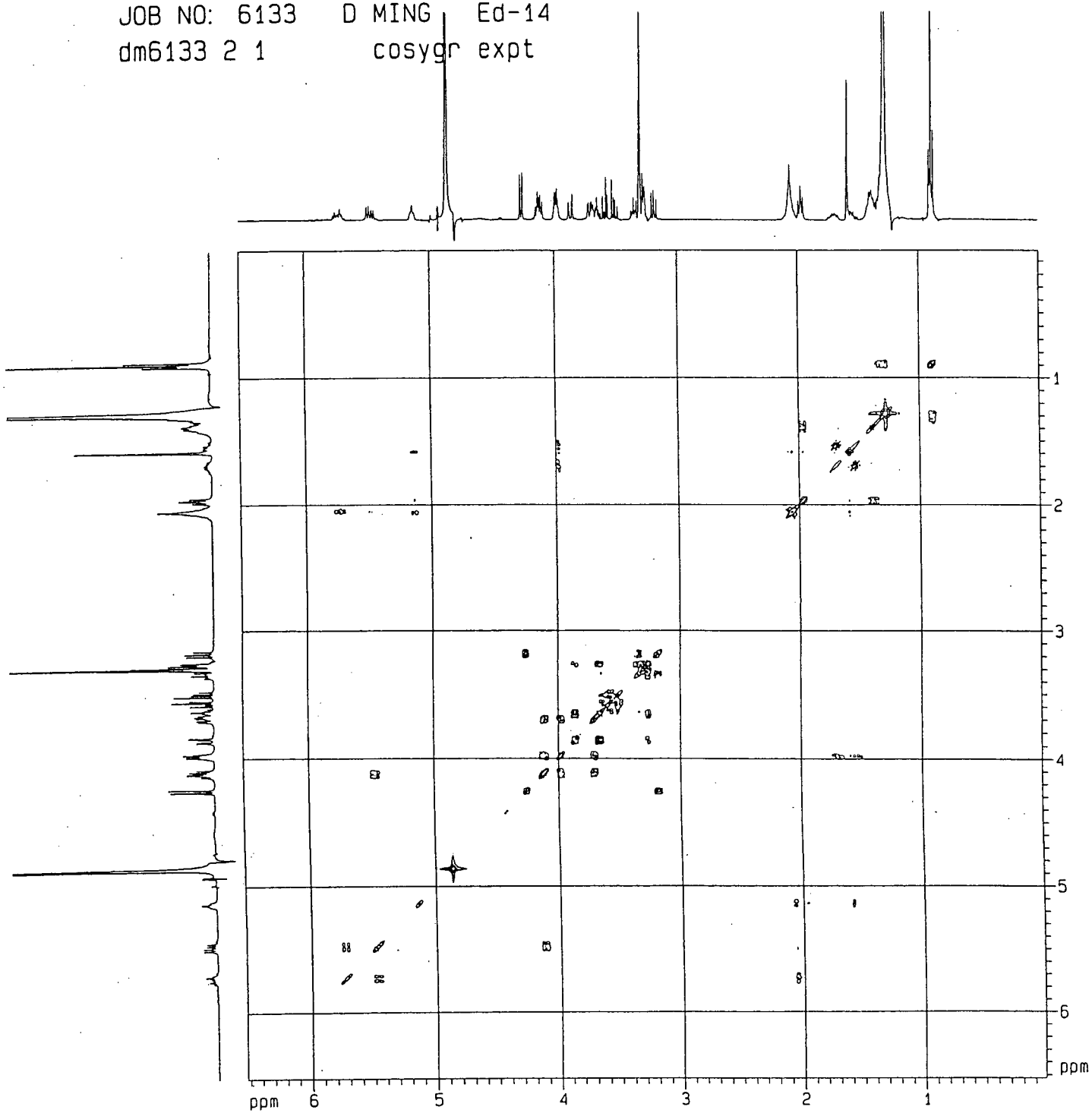
F2 - Processing parameters

SI	32768
SF	400.1300115 MH
WDW	EM
SSB	0
LB	0.20 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	20.00 cm
CY	12.50 cm
F1P	10.000 pp
F1	4001.30 Hz
F2P	-0.200 pp
F2	-80.03 Hz
PPMCM	0.51000 pp
HZCM	204.06630 Hz

JOB NO: 6133 D MING Ed-14
dm6133 2 1 cosygr expt



Current Data Parameters

NAME	dm6133
EXPNO	2
PROCNO	1

F2 - Acquisition Parameters

Date_	20010606
Time	9.48
INSTRUM	av400
PROBHD	5 mm BBI 1H-
PULPROG	cosygp
TD	1024
SOLVENT	MeOH
NS	16
DS	16
SWH	2604.167 Hz
FIDRES	2.543132 Hz
AQ	0.1966580 sec
RG	812.7
DW	192.000 usec
DE	6.00 usec
TE	300.0 K
d0	0.00000300 sec
D1	1.00000000 sec
d13	0.00000300 sec
D16	0.00010000 sec
IN0	0.00038400 sec

======== CHANNEL f1 ========

NUC1	1H
P0	5.00 usec
P1	10.00 usec
PL1	4.30 dB
SFO1	400.1313200 MHz

====== GRADIENT CHANNEL =====

GPNAM1	SINE.100
GPNAM2	SINE.100
GPX1	0.00 %
GPX2	0.00 %
GPY1	0.00 %
GPY2	0.00 %
GPZ1	10.00 %
GPZ2	10.00 %
P16	1000.00 usec

F1 - Acquisition parameters

ND0	1
TD	512
SFO1	400.1313 MHz
FIDRES	5.086263 Hz
SW	6.508 ppm

F2 - Processing parameters

SI	1024
SF	400.1300115 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0
PC	1.00

F1 - Processing parameters

SI	1024
MC2	QF
SF	400.1300115 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

2D NMR plot parameters

CX2	14.00 cm
CX1	14.00 cm
F2PLO	6.524 ppm
F2LO	2610.57 Hz
F2PH1	0.016 ppm
F2HI	6.41 Hz
F1PLO	6.524 ppm
F1LO	2610.57 Hz
F1PH1	0.016 ppm
F1HI	6.41 Hz
F2PPMCM	0.46488 ppm/cm
F2HZCM	186.01190 Hz/cm
F1PPMCM	0.46488 ppm/cm
F1HZCM	186.01190 Hz/cm