THE EFFICACY AND PHYTOTOXICITY OF *XENORHABDUS BOVIENII* A2 METABOLITES AS AGRICULTURAL FUNGICIDES

by

Kong Kueen Ng

B. Sc. (Honours), National University of Malaysia (UKM), 1991

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

Master of Pest Management

in the Department

of

Biological Sciences

SIMON FRASER UNIVERSITY

June 1995

APPROVAL

Name: **KONG KUEEN NG**

Degree: **Master of Pest Management**

Title of Thesis:

THE EFFICACY AND PHYTOTOXICITY OF XENORHABDUS BOVIENII A2 METABOLITES AS AGRICULTURAL FUNGICIDES

Examining Committee:

Chair: Dr. Charles Boone, Assistant Professor

Dr. J. M. Webster, Professor, Senior Supervisor
Department of Biological Sciences, SFU

Dr. J. E. Rahe, Professor,
Department of Biological Sciences, SFU

Mr. D. Ormrod, Plant Pathologist
B. C. Ministry of Agriculture, Fish and Food
Public Examiner

Date Approved 12 June, 1995

PARTIAL COPYRIGHT LICENSE

Title of Thesis/Project/Extended Essay

THE EFFICACY AND PHYTOTOXICITY OF XENORHABDUS BOVIENII A2

METABOLITES AS AGRICULTURAL FUNGICIDES

Author: _____
(signature)

KONG KUEEN NG
(name)

23 JULY 1995
(date)

Abstract

Xenorhabdus bovienii A2 is a bacterial symbiont of *Steinernema carpocapsae*, which is an entomopathogenic nematode currently used to manage some insect pests. This bacterium produces a broad spectrum of antimicrobial metabolites. Experiments were done to test the antimycotic activity and phytotoxicity of the antimicrobial metabolites of *X. bovienii* on selected plant pathogenic fungi on Petri plates and plants, and thereby demonstrating the potential of these substances as agricultural fungicides. Three fractions, organic (ABT001), 'whole' (ABT002), and aqueous (ABT003), obtained from the supernatant of 96-h-old *X. bovienii* culture in tryptic soy broth, were tested. ABT001 at 0.1 mg/ml was fungicidal against *Phytophthora infestans* on rye agar but only partially inhibited *Pythium ultimum* and *Rhizoctonia solani* on 0.39 % PDA in Petri plate tests. Both ABT002 and ABT003 were fungicidal against *P. infestans* at 1.0 mg/ml but not against *P. ultimum* and *R. solani*. In potted plant tests, ABT001 was applied to the foliage of 4- to 5-week-old potato plants (cv. Norchip) and potted grass (mixture of 40 % ryegrass, 40 % fescue and 20 % bluegrass) as a protectant spray at concentrations of 5.0 and 10.0 mg/ml (w/v), and 2.0, 10.0 and 20.0 mg/ml (w/v), respectively. ABT002 was tested on only potato plants at 10.0 and 50.0 mg/ml (w/v). Against *P. ultimum*, ABT001 was coated on bush bean seeds (cv. Venture-Blue Lake) at rates of 0.3, 1.5 and 7.5 mg/g of seed (w/w) with diatomaceous earth and methyl cellulose. When potted potato plants were assessed 7 d after inoculation, the 10.0 mg/ml ABT001 and 50.0 mg/ml ABT002 treatments significantly ($P < 0.05$) inhibited late blight development on inoculated potato leaflets to 4.0 and 0 % respectively, which was not significantly different ($P > 0.05$) from that of the 10.0 mg/ml (w/v) chlorothalonil treatment. At lower concentrations, 5.0 mg/ml ABT001 and 10.0 mg/ml ABT002

significantly ($P < 0.05$) decreased late blight infection to 24.0 and 58.0 %, respectively and reduced the size of the blighted lesions on inoculated leaflets when compared with those of the controls. ABT001 treatments provided incomplete protection against *R. solani* and *P. ultimum* on potted grass and bush bean seeds, respectively. Phytotoxicity was observed on all test plants treated with ABT001, and the phytotoxic damage increased proportionally with increasing concentrations. The visible phytotoxic responses on potato plants treated with up to 10.0 mg/ml ABT001 or 50.0 mg/ml ABT002 were not permanent as treated plants grew away from the phytotoxic damage. The selectivity of ABT001 against the three test fungi, its phytotoxic properties and the potential of the active metabolites from *X. bovienii* culture supernatant in future fungal disease control are discussed.

Dedication

To my parents

for giving me a chance to be a part of this beautiful but challenging world.

Acknowledgments

I would like to express my appreciation to my senior supervisor, Dr. J.M. Webster, for his guidance and support throughout my project and for his critical review of this manuscript. I would also like to extend my gratitude to Dr. J.E. Rahe for his invaluable ideas and encouragement. I would like to thank Dr. Jianxiong Li for sharing techniques and materials and for his advice on extraction processes, Genhui Chen for his invaluable ideas and suggestions and Bruce Leighton for his assistance in potting and plant maintenance.

I would like to acknowledge the National Research Council contract with Agro BioTech Inc. and the British Columbia Agriculture Research Council contract with the British Columbia Ministry of Agriculture, Fisheries and Food for their financial support. This project was supported also through the award of the H.R. MacCarthy Graduate Bursary, 1994 and 1995 and the Dr. J. Yorston Memorial Graduate Scholarship in Pest Management.

Table of Contents

List of Tables

List of Figures

1.0 INTRODUCTION

Plant diseases have been a problem for farmers ever since agriculture began. The expansion of world populations has proportionally increased the demand for food, and decreased the amount of land available for crop production. Intensification of and continuous cultivation have resulted in the increasing prevalence of disease and an ever increasing demand for improved technology to increase the quality and quantity of crop yields. Empirical approaches to chemical disease control have been practiced since ancient times, in which concoctions consisting of salt brine, sulfur, lime, ashes, and salts of copper, mercury, and arsenic have been used to treat tree wounds and to combat the smuts and rusts of our crops. Major advances in organic chemistry (1920-1940) resulted in the synthesis of a vast array of compounds, many of which were shown to have fungicidal activity (Eckert, 1988). The organic fungicides era, which started around 1934, has given rise to some outstanding accomplishments. By 1985, more than 370,000 metric tonnes of pesticides, 10 % of which were fungicides, were produced annually in the United States (US) alone (Anonymous, 1987b; Jewell, 1987).

This major agro-industrial development has undergone dramatic changes in the last 20 years. The 1970s were characterized by steady growth in agricultural production driven by strong demand, high commodity prices, high economic growth and marked improvements in agricultural productivity associated with a worldwide agrochemical growth, averaging 6.3 % per annum in real terms (Finney, 1988). In the early 1980s, world trade and economic growth slowed and, for the first time, agricultural production exceeded demand in OECD countries. Consequently, the 1980s were characterized by commodity surpluses, low commodity prices, declining farm incomes, increased government subsidy burdens

and conflicts between governments over subsidy policies (Finney, 1988). In some parts of the world, as income derived from agriculture declined, there was a reduction in the area of land planted, and a corresponding decrease in the demand for agrochemical products (Finney, 1988). In the US, the area planted to food and feed grains in 1987 was estimated to have fallen by 10 % compared with the previous year (Lethbridge, 1989 see Anonymous, 1987a). In the late 1980s, the agrochemical industry saw a second decline in agrochemical history, this time of 1 % and following only 4 years after the first (Finney, 1988). In contrast to this decline in the agrochemical industry, the world population continued to increase, and did so at the rate of 200,000 per day (Finney, 1988). This, combined with the need for an equitable amount of food per person, suggested that the food supply must increase by 75 % by the year 2000 in order to feed a world population of 6 billion (Blaxter, 1986).

Worldwide, an estimated 67,000 different pest species attack agricultural crops, of which approximately 50,000 species are plant pathogens (Anonymous, 1960). Therefore, it is not surprising that an estimated 37 % of all crops is lost annually to pests; 13 % to insects, 12 % to pathogens, and 12 % to weeds, in spite of the use of pesticides and nonchemical controls (Pimentel, 1985). Crop diseases alone cause a worldwide annual loss in production of 13-20 %, representing US$50 billion (James, 1981). As well, it was estimated that if pesticide use were withdrawn there would be a US$10 billion increase in crop losses (including added cost of employing alternative controls) in the US alone (Pimentel, 1985). The agrochemical industry thrives because its products are in demand, and this demand has been created in part by the inadequacies of alternative forms of pest control. It is a realistic premise that the demand for food will continue to increase, and equally, that the benefits of agrochemicals and of the continuing need for them and/or forms of pest control must be recognized (Griffiths, 1988).

The agrochemical market overall is now approaching maturity and it is generally agreed that there are fewer major unexploited technical opportunities for agrochemicals than heretofore (Finney, 1988). As well, the increasingly strict legislative regime concerning environmental factors has substantially increased the costs and the timescale of new product development and registration (Finney, 1988) and diminished the tendency to apply large quantities of chemical pesticides. Long-term survival in the agrochemical business is dependent on the discovery of new compounds, which requires a significant commitment to research and development. With new legislation and environmental safety requirements, the current discovery rate is estimated to be in the region of 1 for every 20,000 (and rising) compounds screened, compared to 1 in every 5,000 compounds screened 20 years ago (Rowe and Margartis, 1987). Coupled with this, the rate of introduction of new, active compounds has fallen from an average of nine per annum in the 1970s to an average of seven per annum in the 1980s. Consequently, it is becoming increasingly difficult to recover the considerable costs of research and development through the introduction and sale of new products. The cost of bringing a new product to the market can be as high as $US80 million (Lethbridge, 1989 see Anonymous, 1987a), where the contribution of the registration costs to this figure is estimated at $US20 million and rising (Anonymous, 1985).

Despite increasing public pressure for the decreased use of chemical pesticides on food crops, there is a need for new agrochemical products to satisfy changing technical, environmental, user and economic demands. The need is mounting, especially with the increasing number of target organisms developing resistance to pesticides, and the vacuum created by products withdrawn from the market on environmental or safety grounds. In general, future products will have to be highly active and economic, suitable for use in integrated pest management

programs, and have substantial safety margins for the user, environment and the consumer of the treated crops. There is strong competition among agrochemical companies to develop novel fungicides, and this is demonstrated amply by the number of new fungicides reported at the Brighton Crop Protection Conference, held every two years (Table 1).

Over the last two decades, studies of microorganisms as biological control agents against fungal diseases of plants have been numerous. Some of the most intensively studied fungal models are *Trichoderma* spp. and *Gliocladium* spp. (Papavizas, 1985; Papavizas and Lewis, 1988 and 1989), *Coniothyrium minitans* and *Pythium oligandrum* (Whipps *et al.*, 1993), and *Sporidesmium sclerotivorum* (Adams, 1990; Adams and Ayers, 1982), and the bacterial models are *Bacillus subtilis* (Rodgers, 1989), *Pseudomonas fluorescens* (Currier *et al.*, 1988) and *Streptomyces griseoviridis* (Yamaguchi, 1987).

During the past 10 years, several commercial biocontrol preparations have been developed for the control of soil-borne plant diseases (Table 2). However, the use of these organisms to control fungal disease problems on plants on a commercial scale has so far been very limited. The world agrochemical market in 1991 was estimated to be valued at $US26,800 million, with fungicide sales amounting to 21 % of this total (Powell and Jutsum, 1993). However, penetration of this overall market by biological products has been limited to less than 0.5 % or only $US120 million of sales per annum, and most of this is in the form of bio-insecticides. Sales of biofungicides currently amount to around $1 million per annum. Commercial success of such naturally derived biofungicides is limited. *Pseudomonas fluorescens* was developed and sold by Ecogen as 'Dagger', for controlling damping-off in cotton, but has had to be withdrawn because of poor shelf-life of the product (Powell and Jutsum, 1993).

Table 1: Examples of new fungicides reported at the Brighton Crop Protection Conference in 1988 and 1990.

Class of Fungicide	**Company**	**Activity**	**Fungal Target**	**Reference**
Dimethomorph (CME 151)	Shell Forschung GmbH	Systemic	Peronosporaceae *Phytophthora*	Albert *et al.* (1988)
Triazole (RH 7592)	Rohm and Haas	Systemic Protectant	Ascomycetes Deuteromycetes Basidiomycetes	Driant *et al.* (1988)
Triazole (CGA 169374)	Ciba-Geigy	Systemic	Ascomycetes Deuteromycetes Basidiomycetes	Ruess *et al.* (1988)
Triazole (BAS 480 F)	BASF AG	Systemic Protectant	Ascomycetes Deuteromycetes Basidiomycetes	Ammermann *et al.* (1990)
Triazole (SSF-109)	Aburahi Laboratories	Systemic Protectant	*Botrytis cinerea*	Murabayashi *et al.* (1990)
Hexaconazole	ICI Agrochemicals	Systemic Protectant	Ascomycetes Deuteromycetes Basidiomycetes	Waller *et al.* (1990)
Phenylamide (CGA 80000)	Ciba-Geigy	Systemic	Peronosporales	Margot *et al.* (1988)
Thiophene (DU 5110311)	Duphar B.V.	Non-systemic	Ascomycetes Deuteromycetes Basidiomycetes *Pythium* spp.	Hofman *et al.* (1990)
Benzamide (ICIA0001)	ICI Agrochemicals	Systemic	Oomycetes	Heaney *et al.* (1988)

Table 2: Commercial formulations of biocontrol agents effective against plant pathogens (Lewis and Papavizas, 1991).

Name	Antagonist	Target pathogen/host	Source
BINAB-T	*Trichoderma* sp.	*Chondrostereum purpureum*/ trees *Endothia parasitica*/chestnut *Verticillium malthousei*/ mushroom	Bio-Innovation AB, Sweden
Dagger G	*Pseudomonas. fluorescens*	*Pythium ultimum*/cotton *Rhizoctonia solani*/cotton	Ecogen Inc., US
Galltrol	*Agrobacterium radiobacter*	*Agrobacterium tumefaciens* /crucifers	AgBio Chem Inc., US
Mycostop	*Streptomyces* sp.	*Alternaria brassicicola*/ vegetables	Kemira Oy, Finland
Norbac 84-C	*Agrobacterium radiobacter*	*Agrobacterium tumefaciens*/trees	New BioProducts Inc., US
P.g. Suspension	*Phlebia gigantea*	*Heterobasidium annosum*/ trees	Ecological Labs. Ltd., UK
Polygandron	*Pythium oligandrum*	*Pythium ultimum*/sugar-beet.	Vyzkummy ustov Czechoslovakia

The use of microbial products to protect crops against plant pathogenic bacteria originated in Europe and the United States, with the application of antibiotics such as streptomycin, tetracycline and chloramphenicol. Cycloheximide and griseofulvin were introduced later for the prevention and eradication of fungal plant diseases (Eckert, 1988). Subsequently, many attempts were made to find microbial products to control plant diseases but most compounds failed because of their instability in the field or their phytotoxicity. In Japan, a number of secondary metabolites produced by fermentation have been commercialized as fungicides (see Table 3). Their prime targets are rice diseases, with the two rice blast fungicides, blasticidin (produced by Nihon Nohyaku; derived from *Streptomyces griseochromogenes*) and kasugamycin (produced by Hokko Chemicals; derived from *S. kasugaensis*), as the major products (Yamaguchi, 1987). Even though these microbial fungicides are not new (discovered in the 1960s and the 1970s), they have not been commercialized in other parts of the world (Lange *et al.*, 1993), presumably because of concern that their wide use might create resistant strains among medically importance microorganisms and/or the high cost of production.

Natural products are well known to have a range of useful biological properties and have been used by man in many ways over thousands of years. They have been used to solve agricultural problems ever since agriculture began and now are playing an important role in the discovery of modern agrochemicals (Pillmoor *et al.*, 1993). Microbial products for example are unique, in that potentially they can be exploited either as leads for further chemical synthesis or as commercial products in their own right following extraction directly from the producing organism (Beautement *et al.*, 1991; Pillmoor *et al.*, 1993). Leads for chemical synthesis are undoubtedly the preferred route for a company with a synthesis base.

Table 3: Examples of fungicidal products that are based on metabolites produced by Actinomycetes (Yamaguchi, 1987).

Producing organism	Compound	Target disease
Streptomyces caespitosus-detoxicus	Blasticidin	Rice blast
S. hygroscopicus	Validamycin	Sheath blight on rice
S. kasugaensis	Kasugamycin	Rice blast
S. aureochromogenes	Polyoxin (A-K)	Sheath blight on rice
Streptoverticillium rimofaciens	Mildiomycin	Powdery mildew

Two recent examples of natural fungicides, strobilurins and oudemansins, have generated much interest (Anke *et al.*, 1990; Beautement *et al.*, 1991). These compounds are derived primarily from the *Basidiomycetes* complex, especially *Collybia sensu lato* (i.e. *Xerula* sp., *Oudemansiella mucida*, *Strobilurus tenacellus*) (Anke *et al.*, 1979, 1983). Over 100 primary patents have been filed, with some twelve companies involved, and the prospects for the commercial exploitation of this type of chemistry appear to be good in view of reports at the 1992 British Crop Protection Conference (Ammermann *et al.*, 1992; Godwin *et al.*, 1992).

Of equal interest is another group of antibiotics, the pyrrolomycins. The fungicidal metabolite, pyrrolnitrin and its related compounds, isolated from *Pseudomonas pyrrocinia* was the starting point for a synthesis program at Ciba Geigy that resulted in the discovery of fenpiclonil, a fungicide for cereal seed treatment. It will be marketed under the trade name 'Beret®' (Nevill *et al.*, 1988). Another new non-systemic phenylpyrrole fungicide developed from pyrrolnitrin, which provides high level, broad-spectrum activity, is proposed as a foliar fungicide and for seed treatment. It will be marketed under the trade name of 'Saphire®' (Gehmann *et al.*, 1990).

A bacterium that is now receiving a lot of attention is *Xenorhabdus*. It was suggested three decades ago (Dutky *et al.*, 1964) that these bacteria produced a wide spectrum antibiotic, but it was not until the early 1980s that the bioactivity of these bacteria were tested against microorganisms (Poinar *et al*, 1980; Akhurst, 1982). The first chemical isolation and structural assignment of an antibiotic compound from these bacteria was conducted by Paul *et al.* (1981), and since then this bacterium has been a focus for antibiotic research by groups of researchers in Australia (Dr. N.E. Akhurst at CSIRO), the US (Dr. K. Nealson at University of

Wisconsin-Milwaukee) and Canada (Dr. J.M. Webster at Simon Fraser University).

Xenorhabdus spp. belong to a group of unusual entomopathogenic, Gram-negative Enterobacteriaceae. Morphologically, they are asporogenous, rod-shaped bacteria with a highly variable cell size within and between cultures, ranging from 0.3 by 2.0 μm to 2.0 by 10.0 μm and occasionally with filaments 15-50 μm in length, motile and peritrichously flagellated. These bacteria are facultatively anaerobic, and except for *X. nematophilus*, all identified *Xenorhabdus* spp. produce pigment (Akhurst and Boemare, 1988)

Xenorhabdus spp. are mutualistically associated with insect-pathogenic nematodes of the family Steinernematidae (Thomas and Poinar, 1979). Each species of entomopathogenic nematode has a specific, natural association with one *Xenorhabdus* species, though a *Xenorhabdus* sp. may be associated with more than one nematode species (Akhurst and Boemare, 1988) (Table 4). *Xenorhabdus bovienii* was initially identified and classified as a subspecies of *X. nematophilus* before it was elevated to species status. It is associated with *S. feltiae*, *S. affinis*, *S. kraussei*, *S. intermedia*, and some unidentified *Steinernema* spp. (Akhurst and Boemare, 1988). The bacterial symbiont is carried monoxenically in the intestine of the infective-stage, juvenile nematode (IJ). The IJ penetrates an insect host and migrates to the hemocoel where it voids the bacteria. The bacteria proliferate and kill the host, and establish suitable conditions for their own growth and for nematode reproduction by providing nutrients and inhibiting the growth of fungi and other bacteria (Thomas and Poinar, 1979). This capability of killing insects has resulted in this nematode-bacterial complex being developed as a commercial biological control agent.

Akhurst (1980) demonstrated that *Xenorhabdus* produces two colony types. The type that is commonly isolated from the IJ intestine is the primary form

Table 4: *Xenorhabdus* species and their nematode associates.

***Xenorhabdus* species**	**Nematode associate**	**Reference**
X. beddingii	*Steinernema* sp. M isolate	Akhurst and Boemare (1988)
	Steinernema sp. N isolate	Akhurst and Boemare (1988)
X. bovienii	*S. feltiae* (=*bibionis*)	Akhurst and Boemare (1990)
	S. kraussei	Akhurst and Boemare (1988)
	S. affinis	Akhurst and Boemare (1988)
	S. intermedia	Akhurst and Boemare (1990)
	Steinernema sp. F3 isolate	Akhurst and Boemare (1990)
	Steinernema sp. F9 isolate	Akhurst and Boemare (1990)
X. japonicus	*S. kushidai*	Nishimura *et al.* (1994)
X. nematophilus	*S. carpocapsae*	Akhurst and Boemare (1990)
X. poinarii	*S. glaseri*	Akhurst and Boemare (1988)
	Steinernema sp. NC513 isolate	Akhurst and Boemare (1988)
Xenorhabdus sp.#	*S. rara*	Poinar *et al.* (1988)
Xenorhabdus sp.#	*S. cubana*	Mracek *et al.* (1994)
Xenorhabdus sp.#	*S. scapterisci*	Aguillera *et al.* (1993)
Xenorhabdus sp.#	*S. ritteri*	Boemare *et al.* (1993)
Xenorhabdus sp.#	*S. anomali*	Boemare *et al.* (1993)
Xenorhabdus sp.#	*S. neocurtillis*	Nguyen and Smith (1992)
Xenorhabdus sp.#	*S. riobravis*	Cabanillas *et al.* (1994)

Undescribed bacterial species

(phase one). It is unstable *in vitro*, and occasionally *in vivo*, and changes to the secondary form (phase two). Generally, phase one variants of *X. nematophilus*, *X. bovienii*, and *X. beddingii* differ from phase two in dye adsorption, pigmentation, production of antibiotics and lecithinase (Boemare and Akhurst, 1988), and the presence of proteinaceous inclusion bodies (Couche *et al.*, 1987). Morphologically, phase one colonies are generally mucoid and difficult to disperse in liquid, whereas phase two colonies are non-mucoid and are easily dispersed (Boemare and Akhurst, 1988). The phase one of *X. bovienii* absorbs neutral red and forms brown colonies on MacConkey agar and it absorbs bromothymol blue and forms dark blue colonies on amended agar media (Akhurst and Boemare, 1988).

Antimicrobial metabolite production seems to be a crucial feature of all species of *Xenorhabdus* (Poinar *et al.*, 1980; Akhurst, 1982, 1986). The strong and wide ranging antibiotic nature of these metabolites may have evolved due to the unusual symbiotic relationship with the nematode and the special nature of the bacterial growth habitat in an insect cadaver. The antibiotic metabolites prevent putrefaction of the cadaver and growth of microorganisms detrimental to growth and development of *Xenorhabdus* and *Steinernema* (Dutky, 1974). The range of antibiotic activity varies between species (Akhurst, 1982), which suggests that each species produces more than one antibiotic, and that the nature of the antibiotics varies with species.

A variety of antibiotics have been isolated from different species and strains of *Xenorhabdus* (Table 5). Each strain examined has one or two classes of antibiotic, but it is unlikely that the whole spectrum of antibiotics produced by any one strain is known. Li *et al.* (1995) isolated and identified two major classes of antibiotic, the indole and dithiolopyrrolone, from *X. bovienii*. Interestingly, they reported that the purified compounds are active in Petri plate bioassays against

Table 5: The antibiotic nature of the metabolic compounds isolated from *Xenorhabdus* spp.

Species	Strain	Active Compound	Nature of Bioactivity	Reference
X. nematophilus	A11	Xenocoumacin 2	Antibacterial Antiulcer	McInerney *et al.* (1991a).
X. bovienii	T319	Xenorhabdin 1 Xenorhabdin 2 Xenorhabdin 3	nt Antibacterial Insecticidal nt	McInerney *et al.* (1991b).
	Umea	Xenorhabdin 1 Xenorhabdin 2 Xenorhabdin 4 Xenorhabdin 5	nt Antibacterial Insecticidal nt nt	McInerney *et al.* (1991b).
	R[a]	Indole 1, 2, 4 and 4	nt	Paul *et al.* (1981)
	A2	Indole 1, 2, 3, and 4 Xenorhabdin 5, 6[b], 7 and 8	Antifungal nt	Li *et al.* (1995)
Xenorhabdus sp.	Q1	Xenorhabdin 1 Xenorhabdin 2 Xenorhabdin 3 Xenorhabdin 4 Xenocoumacin 1	nt Antibacterial Insecticidal nt nt Antibacterial Antifungal Antiulcer	McInerney *et al.* (1991b). McInerney *et al.* (1991a).

[a] Strain R originally was classified as *X. nematophilus* before a DNA homology study by Grimont *et al.* (1983) showed it to be *X. bovienii*.

[b] Xenorhabdin 5 and 6 isolated by Li *et al.* (1995) are identical to Xenorhabdin 4 and 5 respectively, isolated by McInerney *et al.* (1991b).

nt, not tested

fungi of medical and agricultural importance. The indole derivatives were shown to have significant activity against *Botrytis cinerea* and *Phytophthora infestans*. Although the antimycotic spectrum of the dithiolopyrrolone has not been reported, its related compounds, the pyrrothines, are known to possess a wide range of antimycotic activity (Seneca *et al.*, 1952; Deb and Dutta, 1984). In addition, these metabolic compounds from *Xenorhabdus* are stable at high temperatures (up to 121 °C) (Akhurst, 1982; Maxwell *et al.*, 1994).

Chen *et al.* (1994) tested the spectrum of activity of the antimycotic substances produced by phase one of *Xenorhabdus* spp. on 32 species of fungi from a range of habitats in dual-culture tests. They found that the plant pathogenic fungi, *B. cinerea*, *Ceratocystis ulmi*, *C. dryocoetidis*, *Mucor piriformis*, *Pythium coloratum*, *P. ultimum*, and *Trichoderma pseudokingii* were completely inhibited by all phase one variants, whereas the mycorrhizal fungus *Suillus pseudobrevipes* was not inhibited. They concluded that the antimycotic substances from these bacteria may offer a good opportunity for the control of many species of plant pathogenic fungi.

In nature, *Xenorhabdus* spp. are found only in association with their nematode symbionts and have never been isolated from soil. Chen *et al.* (1994) found that the impact of antibiotics, released from degenerating insect cadavers infected with the entomopathogenic nematodes, on soil microorganisms was short-lived, and suggested that this might be the result of the antibiotics binding to soil particles or to chemical or biological inactivation of these metabolites in soil. In view of the limited interaction between these metabolites and soil microorganisms, and the bacteria's unique habitat during its growth phase, it is not surprising that the antibiotic compounds isolated from *Xenorhabdus* are active against a wide range of microorganisms. So far, the antibiotic effects of *Xenorhabdus* metabolites have been tested only on microorganisms of pharmacological

importance (Akhurst, 1982; McInerney *et al.*, 1991a and 1991b), and it is only recently that their effect on plant pathogenic fungi has been considered (Chen *et al.*, 1994; Li *et al.*, 1995). The collective properties of the antibiotic compounds produced by these bacteria suggest that they could be very useful in plant disease management. However, although these substances inhibit the growth of some plant pathogenic fungi on Petri plate tests no *in vivo* tests have been done. The objective of this research was to test the antimycotic activity and phytotoxicity of *X. bovienii* metabolites on selected plant pathogenic fungi in pot tests to determine the potential of these metabolites as agricultural fungicides.

2.0. GENERAL MATERIALS AND METHODS

2.1. Source and Maintenance of *Xenorhabdus bovienii*

Xenorhabdus bovienii Akhurst (Enterobacteriaceae) strain A2 cultures used in this study were obtained from the culture collection of Dr. J.M. Webster, Department of Biological Sciences, at Simon Fraser University. This bacterium had been isolated from its nematode symbiont, *Steinernema feltiae* Filipjev (Steinernematidae, Rhabdatida) strain A2, which was obtained from topsoil in Merritt, British Columbia. Bacterial cultures were maintained in darkness at 24 °C on 2.3 % nutrient agar (NA) supplemented with 0.004 % triphenyltetrazolium chloride (w/v) and 0.0025 % bromothymol blue (w/v) (NBTA) (Appendix VII). To ensure that it was the phase one variant (as defined by Boemare and Akhurst, 1988) of this bacterial species that was maintained, it was subcultured at 2 week intervals on NBTA using standard, aseptic laboratory procedures. Phase one was distinguished from phase two by its absorption of bromothymol blue from NBTA agar and so produced colonies after 3 to 5 d with a red core overlaid by dark blue and were surrounded by a clear zone. By contrast, phase two produced red or maroon colonies, with no clear zone. So as to minimize the chance of culture loss or contamination and to help ensure consistency of the strain over the long-term, bacteria were grown, suspended in 2.75 % tryptic soy broth (TSB) (Appendix VII) containing 15 % glycerol (v/v), and stored at -20 °C until required, as described by Chen (1992).

2.2. Production and Extraction of Antimycotic Substances

Except where otherwise stated, the production of all antimycotic substances of *X. bovienii* followed the procedures described below.

2.2.1. Bacterial fermentation

Seed culture was prepared by inoculating a loopful of phase one bacteria from a 96 h culture on an NBTA plate into 100 ml of sterile TSB in 250 ml Erlenmeyer flasks. The flasks were plugged with sterile nonabsorbing cotton, covered with aluminum foil and incubated in darkness at 25 °C on an Eberbach rotary shaker, at 130 rpm for 20-24 h. When the optical density (600 nm) and pH readings were approximately 2.0 and 7.0-7.1, respectively bacterial cultures from the 250 ml flasks were mixed thoroughly in a 1,000 ml Erlenmeyer flask to ensure random suspension of the bacterial cells. A 100 ml aliquot of this mixed bacterial culture was transferred aseptically to 900 ml of sterile TSB in 2,000 ml Erlenmeyer fermentation flasks. The flasks were plugged and covered with sterile aluminum foil and incubated in the dark at 25 °C on an Eberbach rotary shaker at 130 rpm. After 96 h incubation at pH 7.5-7.6, the culture was centrifuged (10,000 rpm, 20 minutes, 4 °C) to separate the bacterial cells. The culture supernatant from different flasks was mixed together and stored at 4 °C until required for use.

2.2.2. Lyophilization

Lyophilization was used primarily to concentrate the 96 h culture supernatant for subsequent phytotoxicity test on selected plants. The cool, supernatant was divided into aliquots of 200 ml each in glass tubes and frozen in a -86 °C freezer (Forma Scientific) overnight. The frozen supernatant was lyophilized in a freeze-dryer (Virtis Freezemobile 25EL) for 4 d, and the dried powder collected, weighed and stored in a dry, sealed flask in darkness at room temperature (16-20 °C).

2.2.3. Extraction process

I. Organic fraction

One liter of the cooled, culture supernatant was poured into a 2,000 ml separating funnel (Pyrex) and 500 ml of ethyl acetate (AnalaR®, BDH, Toronto) was added (Figure 1). The funnel was shaken vigorously by hand, the 'mixture' was allowed to separate into a yellowish, ethyl acetate fraction and an aqueous fraction. The aqueous fraction was tapped off into another separating funnel and extracted with another 500 ml of ethyl acetate. Each liter of supernatant was extracted three times with ethyl acetate. Both the ethyl acetate and aqueous fractions were collected and stored at 4 °C for further processing.

The combined ethyl acetate fractions resulting from the bacterial fermentation were dried with anhydrous sodium sulfate, filtered through glass wool and concentrated on a rotary evaporator under vacuum, below 30 °C, to yield a viscous orange-brown, oily substance which was stored at 4°C. All batches of 96 h culture supernatant, extracted similarly with ethyl acetate were assumed to contain the organic soluble, active metabolites from the bacteria and were labeled ABT001.

II. 'Whole' fraction

One liter of the cooled, culture supernatant was passed through a glass chromatographic column (90 x 5 cm) containing approximately 50 cm Amberlite XAD-2 resin (Sigma) suspended in water (Figure 1). The Amberlite XAD-2 column was washed with 3 liters of distilled water, followed by 2 liters of a 1:1:1 mixture of distilled water:butanol (Anachemia, BDH, Toronto):acetic acid (Anachemia, BDH, Toronto). The distilled water:butanol:acetic acid eluate was collected and concentrated on a rotary evaporator under vacuum to yield a viscous brown substance, which was stored at 4 °C. All batches of the 96 h culture

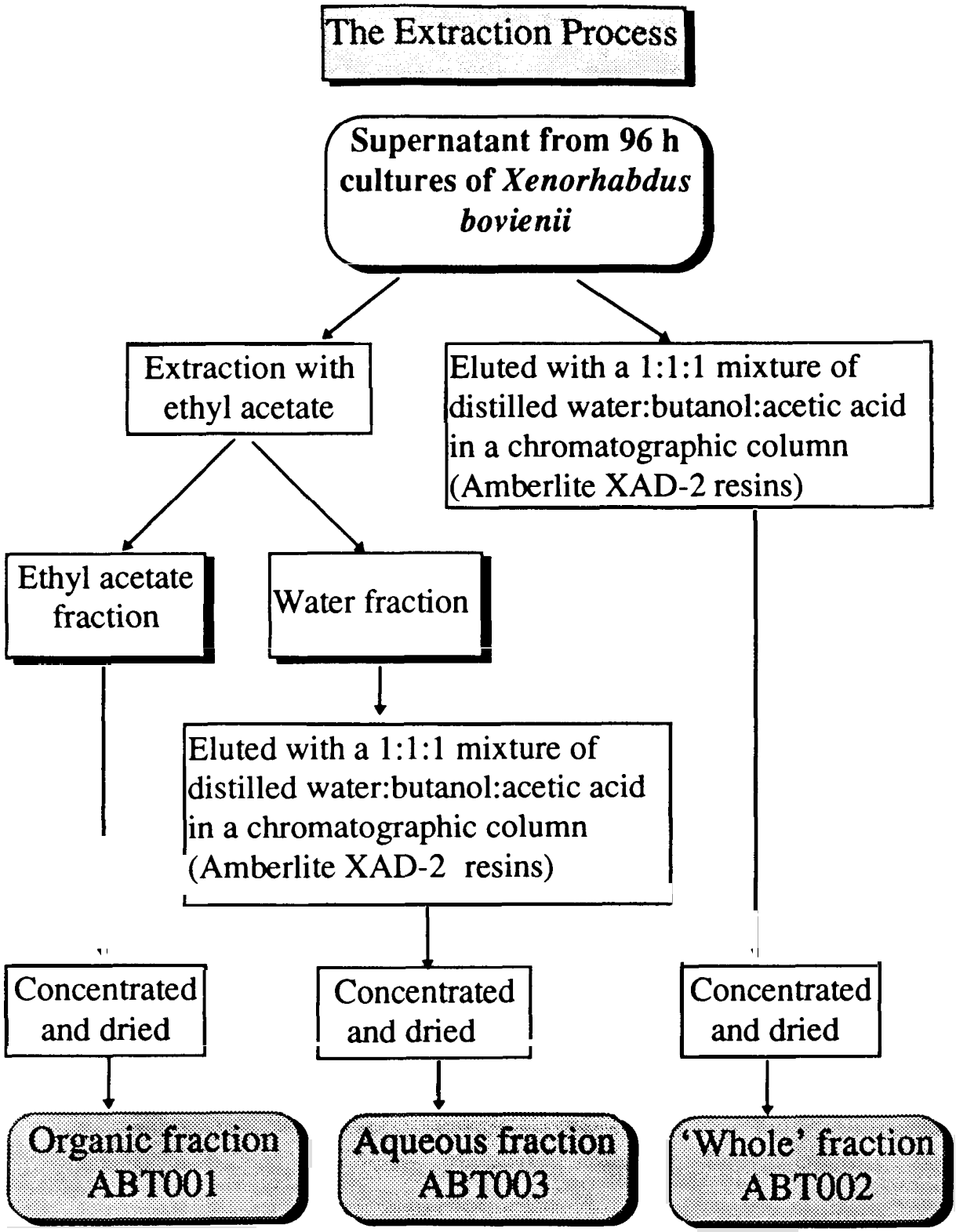


Figure 1: A summary of the extraction process of the 96 h culture supernatant to produce the organic (ABT001), 'whole' (ABT002) and aqueous (ABT003) fractions.

supernatant treated in this way were assumed to contain the majority of the organic and water soluble, active metabolites from the bacteria, and were labeled ABT002.

III. Aqueous fraction

The aqueous fraction from the ethyl acetate extraction process (section 2.2.3-I) was treated with distilled water:butanol:acetic acid (1:1:1) as described in section 2.2.3-II (Figure 1). The concentrated eluate which was a viscous light brown substance was stored at 4 °C. All aqueous fractions treated similarly were assumed to contain the majority of the water soluble, active metabolites from the bacteria, and were labeled ABT003.

2.3. Bioassay of Antimicrobial Activity

All aqueous test solutions were neutralized with 1 M HCl or 1 M NaOH and sterilized by filtration through 0.2 μ-pore filter (Millipore) before being used in the bioassays.

2.3.1. Bacterial Bioassay

Bacillus subtilis was chosen as the standard indicator of antibacterial activity because it is a commonly used species for bioassay of antibiotics and is very sensitive to antibacterial substances produced by *Xenorhabdus* spp. (Poinar *et al.*, 1980; Akhurst, 1982; Maxwell *et al.*, 1994). The *B. subtilis* culture was kindly provided by Dr. J. E. Rahe, Department of Biological Sciences, Simon Fraser University. Upon receipt, the bacteria were cultured on plates of NA in darkness at room temperature (16-20 °C). For long-term maintenance of the stock culture, the bacterial plates were stored at 4 °C.

A standard spore suspension of *B. subtilis* was used for bioassay, and its preparation was a modification of Hewitt and Vincent (1989). Bacterial inoculum was prepared by inoculating a loopful of freshly cultured bacteria into 10 ml of sterile TSB in a 100 ml Erlenmeyer flask. The culture was grown for 24 h at 37 °C, on a rotary shaker at 80 rpm. Aseptically, 1.0 ml of the culture was pipetted into 500 ml of sterile TSB in a 1,000 ml Erlenmeyer flask, and incubated for 10 d under the same environmental conditions as for inoculum preparation. The culture was centrifuged (10,000 rpm, 5 minutes, 4 °C) and washed with sterile distilled water thrice, resuspended in sterile 0.9 % NaCl solution (w/v) and pasteurized at 80 °C in a water bath for 20 minutes. A concentrated spore suspension of $6x10^9$ colony-forming-units (cfu) per ml was prepared from the pasteurized, saline suspension. Aseptically, 1.0 ml of the spore suspension was transferred into Eppendorf tubes and stored at -20 °C until required.

An agar diffusion bioassay (Hewitt and Vincent, 1989) was employed to test for antibacterial activity. Tryptic soy agar (TSA) (Appendix VII) was autoclaved for 15 minutes, cooled in a water bath at 50 °C for 1 hour, and then 10 ml of TSA was injected aseptically into 8.5 cm Petri plates with a sterile 60 ml syringe. After the agar had solidified, the plates were stored at 4 °C in plastic bags until required. A 100 µl spore suspension (6×10^9 cfu per ml) of *B. subtilis* was pipetted aseptically onto each TSA plate that had been warmed to room temperature (16-20 °C), and it was spread evenly over the agar with a sterile, L-shaped glass rod. The plates were dried on a laminar air flow (1.143×10^{-3} kg/cm^2) bench with their lids opened for 20 minutes. Three wells, each of 0.5 cm diameter, were cut in a triangular arrangement in the inoculated agar with an alcohol-flamed, sterilized cork borer, and the agar plugs were removed with sterile forceps. Into each well, 40 µl of the test solution was pipetted at random, the plates were closed and incubated in the dark at 25 °C.

After 24 h incubation, the plates were examined for clear, circular zones around the wells. Each clear zone was measured across two diameters with hand-held calipers. Antibacterial activity was expressed as units of activity (U), where 1 U was defined as a 1.0-mm annular clearing around the well (see Maxwell *et al.*, 1994).

2.3.2. Fungal bioassay

Botrytis cinerea was chosen as the standard indicator of antimycotic activity because it was found to be very sensitive to antimycotic substances produced by the phase one variants of *Xenorhabdus* spp. (Chen *et al.*, 1994). The *B. cinerea* culture was kindly provided by P. Sholberg of Agriculture Canada, Summerland, and was maintained on 3.9 % potato dextrose agar (PDA) (Appendix VII) at room temperature (16-20 °C), under normal fluorescent light.

A standard spore suspension from a 1-month-old culture of *B. cinerea* on PDA plate was used for each fungal bioassay. A 5.0 ml volume of 0.04 % Tween 20 solution (w/v) prepared in sterile distilled water was pipetted aseptically over the fungal culture, and an L-shaped glass rod was used to dislodge spores from the mycelia. The spore suspension was collected and diluted to a concentration of 10^6 spores per ml by appropriate dilution of the original suspension based on the mean of three spore counts made with the help of a hemocytometer. A fresh spore suspension was prepared each time an antimycotic bioassay was performed.

Potato dextrose agar was autoclaved for 15 minutes and cooled to 45 °C in a water bath. One ml aliquots of freshly prepared spore suspension of *B. cinerea* were pipetted into 8.5 cm Petri plates, and 9.0 ml of the cooled, liquid PDA was injected aseptically into the plates using a sterile, 60 ml syringe. Each plate was rotated five times clock-wise and anti clock-wise to distribute the spores evenly. After the agar had solidified and cooled to room temperature, three wells, each of

0.5 cm in diameter, were cut with an alcohol-flamed, sterilized cork borer, and the agar plugs were removed with sterile forceps. Into each well, 40 µl of the test solution was pipetted randomly, and the plates were covered and incubated at 25 °C in the dark. After 48 h of incubation, the plates were examined for clear, circular zones around the wells. Each clear zone was measured across two diameters with hand-held calipers. Antimycotic activity was expressed as units of activity, where 1 U was defined as a 1.0-mm annular clearing around the well.

2.4. Source and Culture of Test Fungi

Cultures of *P. ultimum* and *Rhizoctonia solani* were provided by R. Descalzo, Department of Biological Sciences, Simon Fraser University and Dr. Tom Shiang, Turf Grass Institute, University of Guelph, respectively. Both fungi were maintained on PDA plates, in darkness, at room temperature (16-20 °C) and subcultured every 2 weeks. The culture of *P. infestans* (A1 mating type) was provided by Dr. Zamir Punja, Department of Biological Sciences, Simon Fraser University. It was maintained on rye agar (Appendix VII) in darkness at 16 °C and subcultured every 4 weeks.

2.5. Preparation of Fungal Inoculum

2.5.1. *Pythium ultimum*

Pythium ultimum inoculum was prepared from 8-day-old culture mats grown in V-8 cholesterol broth (Ayers and Lumsden, 1975) (Appendix VII). Sterile 250 ml Erlenmeyer flasks containing 50 ml of V-8 cholesterol broth were each inoculated with one 0.7 cm diameter mycelial plug from a 1-week-old culture

on PDA plate and incubated in darkness at 20 °C. After 8 d, the mycelial mats were recovered from the flasks, gently washed with distilled water and blotted dry on paper towels (Scott Paper). One gram of mycelial mat was cut into smaller pieces (ca. 4 mm^2), mixed with 100 ml of sterile, 0.08 % Anachemia water agar and macerated in a Sorval blender for 20 s at medium speed. Preliminary tests had shown that 1.0 ml of this milky, mycelial suspension when inoculated on PDA plates would produce 10^4 colony-forming-units (cfu). The above procedures and experimental conditions were followed closely in every preparation so as to ensure the same fungal concentration (10^4 cfu/ml) in each inoculum.

2.5.2. *Rhizoctonia solani*

The method used to produce inoculum was modified from that of Sanders and Cole (1986). A culture medium consisting of 50 g rye grain, 1.0 g $CaCO_3$, and 75 ml water was prepared and autoclaved in 250 ml Erlenmeyer flasks with cotton plugs at 121 °C for 45 minutes. Three, 0.7 cm diameter mycelial plugs from a 1-week-old culture of *R. solani* maintained on PDA were transferred into each flask containing the moist, autoclaved rye grains and incubated for 1 week in darkness at 24 °C. Fungus infected rye grains were used as inoculum.

2.5.3. *Phytophthora infestans*

Cultures of *P. infestans* were grown on plates of rye agar in the dark at 16 °C for 2 weeks. A 0.5 cm diameter agar plug was used as an inoculum.

2.6. Source and Preparation of Test Plants

Except where otherwise stated, all planting material consisted of unsterilized garden mix, with two parts top soil, one part sand, and one part peat in

13 cm diameter plastic pots. Unless stated otherwise, all plants were grown in a Conviron growth chamber with 16 h photoperiod (fluorescent tubes at approximately 3,800 Lux) at 25 °C, watered every other day, and were fertilized twice a week with 0.5 % N-P-K (20:20:20) (w/v).

2.6.1. Bush bean

Bush bean, *Phaseolus vulgaris* cv. Venture-Blue Lake, seeds were purchased from Territorial Seeds (Vancouver, B.C.). They were sown directly into plastic pots of garden mix, 2 seeds per pot, and subsequently thinned to one seedling per pot.

2.6.2. Cucumber

Cucumber, *Cucumis sativus* cv. Calypso, seeds obtained from Dr. Zamir Punja, Department of Biological Sciences, Simon Fraser University, were sown directly into plastic pots of garden mix, 2 seeds per pot, and subsequently thinned to one seedling per pot.

2.6.3. Winter wheat

Winter wheat, *Triticum aestivum* cv. Monopol, seeds were purchased from Buckerfields Co. Ltd. (Abbotsford, B.C). Approximately 15 grains were sown into each of several plastic pots of garden mix and kept in the greenhouse with a 16 h photoperiod at 20 °C. When seedlings were 2-week-old, the number of plants in each pot was reduced to ten, by removing the smaller and/or oversized seedlings.

2.6.4. Tomato

Tomato, *Lycopersicon lycopersicum* cv. Fanstatic, seeds were purchased from Territorial Seeds (Vancouver, B.C.) and sown in a 53 x 27 cm plastic flat of garden mix under standard conditions. Two-week-old seedlings were transferred into plastic pots containing the same garden mix and maintained under the standard conditions.

2.6.5. Grass

Grass seeds, purchased from Richardson Seed Co. Ltd. (Burnaby, B.C.), were a mixture of Sovereign Perennial Ryegrass (40 %), Enjoy Chewing's Fescue (20 %), Vista Creeping Red Fescue (20 %) and Shamrock Kentucky Bluegrass (20 %). Into each 10 cm diameter, plastic pot of garden mix, 0.8 g of grass seeds were sown. Pots were covered with brown paper to retain moisture and speed germination, and placed in a growth chamber at 20 °C. Throughout the growing period grass was maintained at a height of 2.5-3.0 cm above the soil surface by cutting regularly with scissors.

2.6.6. Potato

Seed potato, *Solanum tuberosum* cv. Norchip, was donated by Bill Zylmans, W & A Farms, Richmond, B.C. Potato tubers were taken from the cold room (4 °C) and allowed to sprout in darkness at room temperature (16-20 °C). Sprouted tubers were cut into pieces (40-50 g each) each with one or two sprouts about 1.0-1.5 cm long, and placed 2-3 cm below the soil surface in plastic pots of garden mix and maintained at 16-20 °C. Each pot was thinned to one stem after emergence.

2.7. Statistical Analyses

Except where otherwise stated, all experiments followed a standard randomized design. Statistical analyses were conducted using the SAS statistical package (SAS Institute Inc., Cary, North Carolina). Observations of repeated experiments were subjected to analyses of homogeneity using Student's *t*-test. Where no significant differences were found, data from repeated experiments were pooled together and a combined analysis of variance was performed. Comparison means between different ANOVA cells were calculated using Student-Newman-Keul's test. In all analyses, levels of significance of $P < 0.05$ were used.

3.0. EXPERIMENTS AND RESULTS

3.1. Fermentation Process of *Xenorhabdus bovienii*

3.1.1. Standard growth curve

In order to minimize the qualitative and quantitative variation in the composition of the metabolic products in various fractions, the *X. bovienii* culture was processed at specific time in the growth of the culture. To facilitate determination of the optimum sampling time, a standard growth curve was prepared for X. *bovienii*. The seed culture of *X. bovienii* was prepared as described in section 2.2.1, and the culture's optical density (600 nm) was continually monitored with a spectrophotometer (Spectronic 50). When the readings for the undiluted culture samples reached approximately 2, at the mid-exponential growth phase, the content of these flasks were mixed together in a 1,000 ml Erlenmeyer flask, and the pH was recorded (pH meter 320, Corning). Subsequently, 100 ml of the mixed bacterial culture was added aseptically to 900 ml of TSB in each of the three 2,000 ml Erlenmeyer flasks, and incubated for 144 h under the same conditions as described in section 2.2.1. The flask positions were rotated each day to minimize any positional effect on the shaker. To monitor bacterial growth, the optical density (600 nm) of three, 1.0 ml culture samples taken from each flask at different incubation times (every 2-3 h for the first 24 h and every 6-12 h for the next 5 d), was recorded spectrophotometrically. Concurrently, the pH of each sample was taken. The viable cell count of this bacterium during the fermentation process was determined by the spread plate method. One ml of the culture was pipetted aseptically from each flask into 9 ml of sterile TSB. Three to six serial, tenfold dilutions were performed, depending on the time of the fermentation process. Subsequently, 0.1 ml from each dilution tube was pipetted onto TSA plates, spread evenly with an alcohol flamed,

sterilized, L-shaped glass rod, and incubated at 25 °C. Each dilution was plated thrice, and the number of yellow colonies was counted after 24 h. The optical density, total viable cell count and pH readings were plotted against time.

3.1.2. Antibacterial and antimycotic activity

The production of antibacterial and antimycotic substances by *X. bovienii* was monitored by bioassaying the culture supernatant over 6 d of fermentation. An 8 ml culture sample was collected every 24 h from each of the three culture flasks, and immediately centrifuged for 1 minute in an Eppendorf centrifuge (Brinkmann) to pellet the bacterial cells. One ml of the sample supernatant for each sampling hour from each flask was pipetted into Eppendorf tubes and stored at 4 °C until required for the antibacterial and antimycotic bioassay (section 2.3.1. and 2.3.2). In each bioassay, samples were replicated three times using three plates. As a control, 40 µl of TSB alone was pipetted into the wells. The rest of the supernatant was extracted only once with an equal volume of ethyl acetate. Five ml of the resulting extract were dried on a rotary evaporator under vacuum below 30 °C, and redissolved in 1 ml of methanol. This crude antibiotic extract in methanol was pipetted into a 5 ml glass container, sealed with Parafilm and stored at 4 °C for subsequent antibacterial and antimycotic bioassays. In each bioassay, samples were replicated three times using three plates. As a control, 40 µl of methanol was used. This experiment was repeated once. The diameter of the antibacterial and antimycotic inhibition zones were plotted against fermentation time.

3.1.3. Results

A preliminary laboratory study of *X. bovienii* in 100 ml of TSB in 250 ml flasks showed good bacterial growth in the first 24 h, and the inoculum culture

reached the mid-exponential growth phase 20 h after inoculation. When monitored spectrophotometrically at 600 nm at 20 h, undiluted inoculum culture measured 1.91, and with pH 7.15. Subsequently, when cultured in 2,000 ml flasks no lag phase was observed in the bacterial culture after 100 ml of the inoculum culture was inoculated into the 900 ml of TSB (Figure 2a). The exponential growth phase began immediately from the time of inoculation, continued for about 15 h with a generation time of approximately 2.3 h, and was accompanied by a decrease in pH from 7.45 to minimum of 7.05 at 9 h (Figure 2a). Thereafter, the bacterial growth rate slowed as the culture pH increased. As the growth rate slowed, there was a sharp decrease in the culture viable cell count from more than 6.5 x 10^8 cell/ml at 15 h to 1.7 x 10^8 cell/ml at 20 h before fluctuating within the range of 5-20 x 10^7 cell/ml as fermentation proceeded from 20 to 144 h (Figure 2b). During the stationary growth phase after 20 h, the pH continued to rise and reached 7.98 by 144 h of fermentation. The bacterial culture began to produce a yellow pigment as it entered the stationary phase, and it was readily observed on the inner surface of the flask after 24 h of fermentation.

The antibacterial and antimycotic activity of the culture supernatant increased during the growth of the bacteria, especially during the exponential growth of the first 24 h and thereafter the rate of increase leveled with broad maxima at 72 and 96 h, respectively (Figure 3a and Appendix I). In the organic fraction, the antibacterial activity increased rapidly in the exponential and early stationary phase and leveled off after 48 h of fermentation whereas the antimycotic activity increased more slowly and began to level off at the early-stationary phase (Figure 3b). The organic fraction had the highest antibacterial and antimycotic activity when bioassayed at 96 and 144 h (see Appendix II).

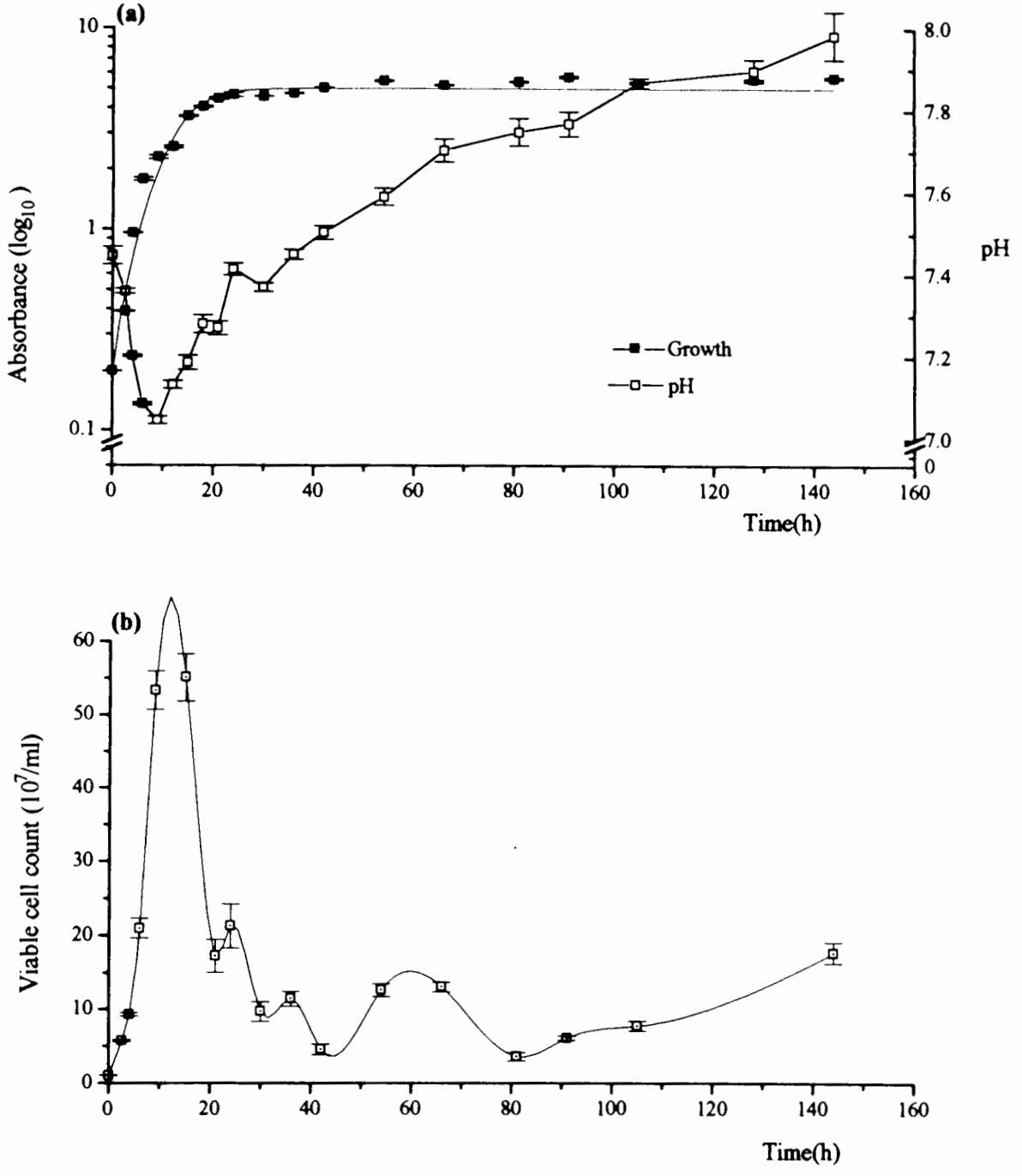


Figure 2: Growth curve of *Xenorhabdus bovienii* showing (a) bacterial growth (absorbance at $OD_{600\ nm}$) and culture pH, and (b) the viable cell count (10^7/ml) of the fermentation culture over time (n = 6) .

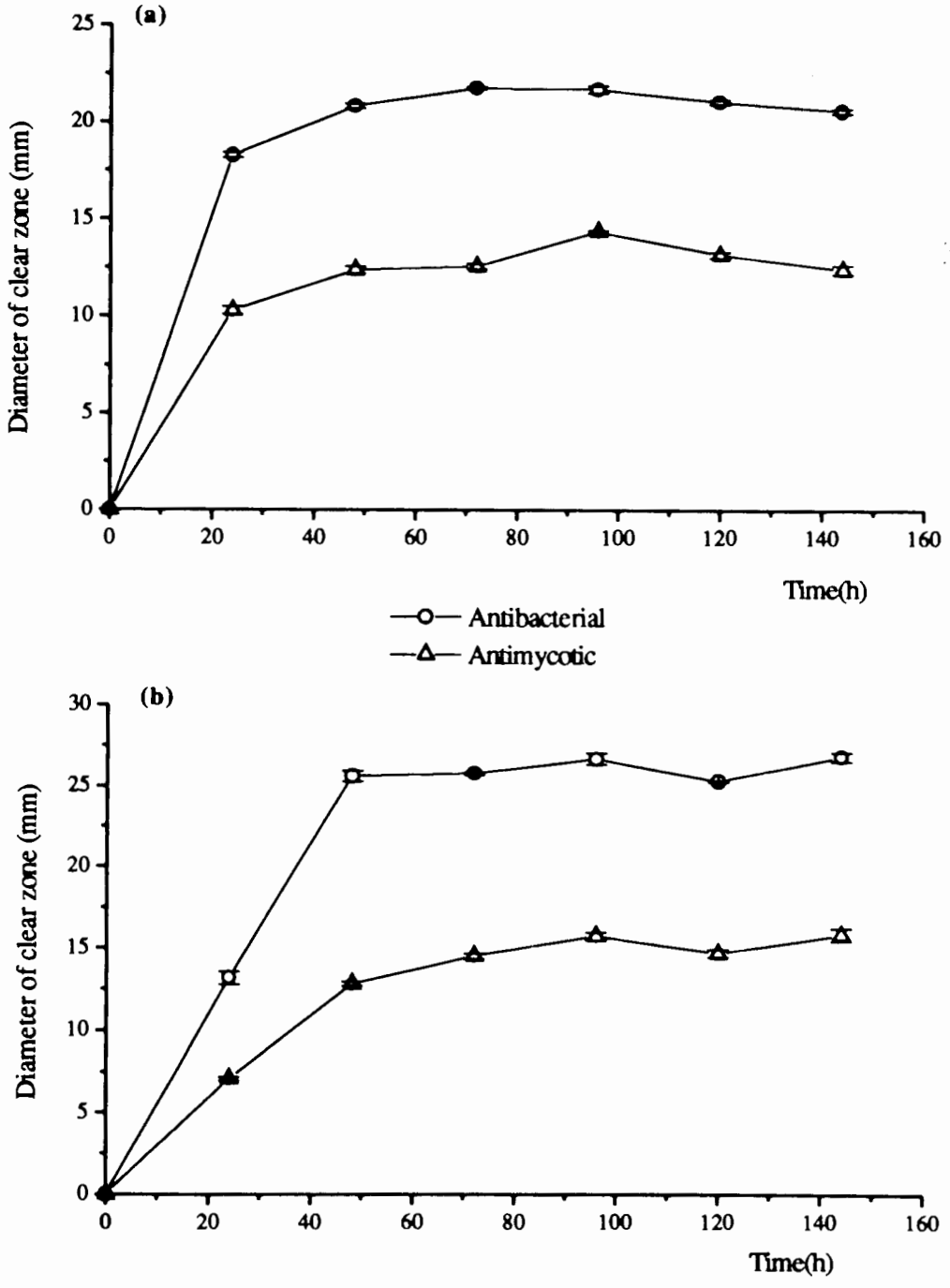


Figure 3: Antibacterial and antimycotic activity of the (a) supernatant and (b) organic fraction of the *Xenorhabdus bovienii* culture (n = 6).

Throughout the duration of this research project, the phase one culture of *X. bovienii* was maintained by subculturing on NBTA plates every 2 weeks. Only the dark blue colonies of the phase one variant were observed on these regularly subcultured plates. The typical red or maroon colonies of the phase two variant were never seen. The culture was monitored by assaying four randomly selected batches of the 96 h culture supernatant over the 10 month period of the project with *B. subtilis*. Results showed no significant difference in the diameter of the cleared zone between three of the four cultures assayed (Table 6). However, there was a significantly smaller clear zone in the third, October, sample when compared with the other three samples.

3.2. Antimycotic Test against Selected Fungi

Unless otherwise stated, the organic (ABT001), 'whole' (ABT002) and aqueous (ABT003) fractions used in this and subsequent experiments were mixtures from several batches of 96 h *X. bovienii* culture produced over a 2 month period.

3.2.1. Organic fraction (ABT001)

A stock solution of ABT001 (100 mg/ml) was prepared by dissolving the dried, organic fraction in a Tween 20 solution. The solvent was made up of 0.04 g Tween 20 in 100 ml of a 1:1 mixture of acetone (Anachemia, BDH, Toronto):distilled water. To determine the effective antimycotic concentration of the organic fraction against specific fungi two serial, tenfold dilutions were made of the filter-sterilized (0.2 μ-pore filter, Millipore) stock solution of ABT001 with the Tween 20 solution. From each dilution, 2.0 ml was thoroughly mixed with 98 ml of liquid rye agar or 0.39 % PDA in a 250 ml Erlenmeyer flask in a

Table 6: Antibacterial activity of 96 h culture supernatant of four randomly selected *Xenorhabdus bovienii* fermentation cultures, expressed as the diameter of the clear zone on a *Bacillus subtilis* plate.

Sample	Diameter of cleared zone (mm)
24 February 1994	21.72 ± 0.14a[#]
2 May 1994	21.68 ± 0.15a
1 October 1994	20.93 ± 0.17b
8 November 1994	21.93 ± 0.08a

[#] Mean ± standard error of the mean, n = 6. Means with same letter are not significantly different ($P < 0.05$).

waterbath at 45 °C. The final concentration of the three dilutions in rye agar or 0.39 % PDA were 0.01, 0.1, and 1.0 mg/ml. Aseptically, 10 ml of the agar was injected into 8.5 cm Petri plates with a sterile, 60 ml syringe. Mycelial plugs (0.7 cm diameter) from 2-week-old cultures of *P. infestans* and 1-week-old cultures of *P. ultimum* and *R. solani* were placed in the center of their respective plates of solidified agar. As controls, plates contained only rye agar or 0.39 % PDA with distilled water or Tween 20 solution. Two commercially formulated fungicides, Bravo 720 (54 % chlorothalonil a.i.) and Apron TL (11.5 % metalaxyl a.i.), were used for comparison. Both fungicides were dissolved in sterile distilled water and mixed thoroughly with the liquid rye agar or 0.39 % PDA. The final a.i. concentration of each commercial fungicide in the agar mixture was 0.1 mg/ml. Inoculated plates were sealed with Parafilm and placed randomly in their respective incubators, 20 °C for *P. ultimum*, 24 °C for *R. solani* and 16 °C for *P. infestans*. The growth of the fungal mycelia was recorded daily by measuring the diameter of the fungal colony with hand-held calipers. The experiment was terminated when mycelium in one of the control plates reached the edge of the Petri plate. Each concentration of ABT001 and the controls was replicated five times, and this experiment was repeated once.

Mycelial plugs from Petri plates that did not show any mycelial growth after the experiment was terminated were transferred to untreated rye agar or 3.9 % PDA to assess whether the antimycotic substances in ABT001 were fungicidal or fungistatic. PDA plates with *P. ultimum* and *R. solani* agar plugs were incubated in their respective incubators for 48 h, whereas rye agar plates with *P. infestans* agar plugs were incubated for 7 d at 16 °C. If the transferred mycelial plug in untreated rye agar or PDA did not show fungal growth, the antimycotic substances were identified as fungicidal. If the transferred mycelial plug showed fungal growth, the antimycotic substances were considered to be fungistatic.

3.2.2. 'Whole' fraction (ABT002) and aqueous fraction (ABT003)

The same procedure as described in section 3.2.1. was followed to determine the strength of the antimycotic activity ABT002 and ABT003 against the three test fungi. However, sterilized distilled water was used as solvent instead of the Tween 20 solution. ABT002 and ABT003 solutions were neutralized with 1 M NaOH before they were filter-sterilized and mixed with their respective agars. The same controls as described in section 3.2.1 were used. Each treatment was replicated five times, and this experiment was repeated once.

3.2.3. Results

The organic fraction, ABT001 completely inhibited fungal growth of *P. infestans* on rye agar at rates of 0.1 and 1.0 mg/ml over 7 d (Figure 4 and 5a). No mycelium was observed around these mycelial plugs inoculated on the treated rye agar and when observed under a dissecting light microscope, the mycelia on these plugs appeared lysed with a layer of exudate around them. When the mycelial plugs were transferred onto untreated rye agar after the experiment was terminated, no fungal growth was observed. Similar observations were noted with the chlorothalonil (0.1 mg/ml) treatment. The 0.01 mg/ml ABT001 treatment significantly slowed fungal growth when compared with that on rye agar or rye agar amended with the solvent. Metalaxyl (0.1 mg/ml) treatment slowed but did not completely inhibit growth of *P. infestans*.

Only the highest concentration (1.0 mg/ml) of ABT001 inhibited growth of *R. solani* and this was comparable with the chlorothalonil treatment after 48 h incubation (Figure 5b). Although no radial growth was recorded around the mycelial plugs of the ABT001 treatment, there were traces of fungal growth on the surface of the plugs. When these plugs were transferred onto PDA plates at the end of the experiment, good fungal growth was observed after the plates were

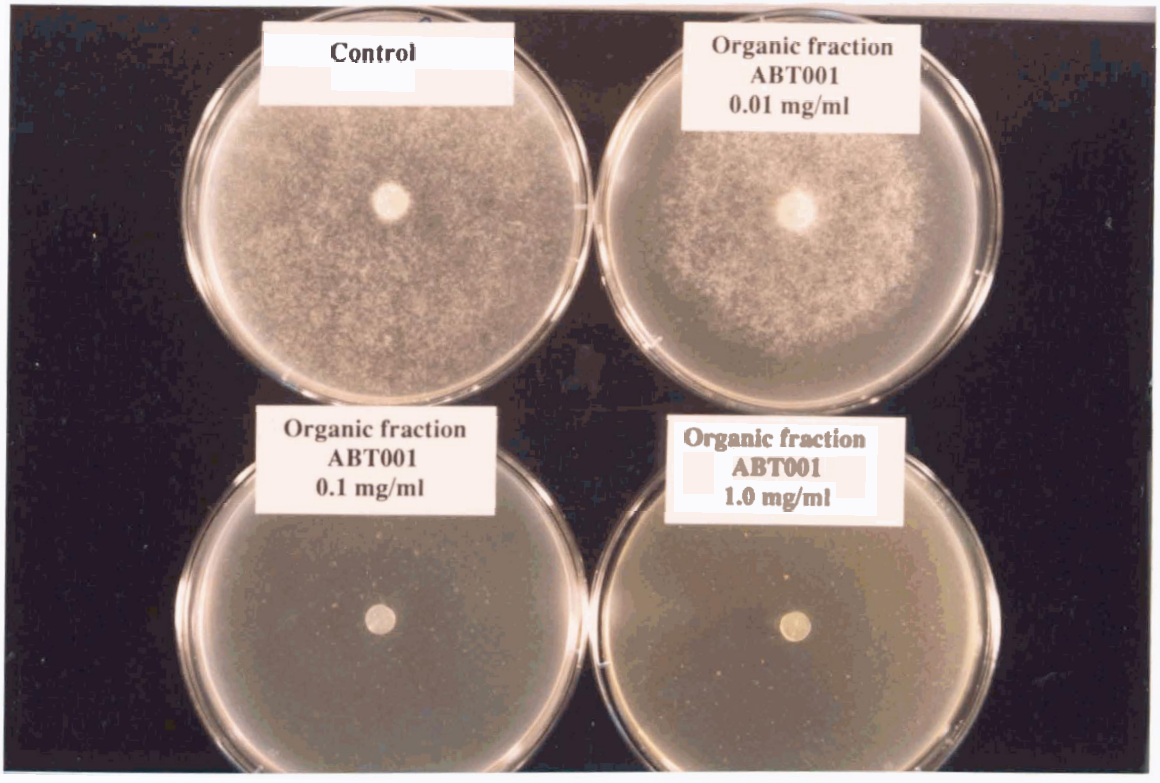


Figure 4: The growth of *Phytophthora infestans* on rye agar treated with 0.01, 0.1 or 1.0 mg/ml ABT001 or with distilled water (control).

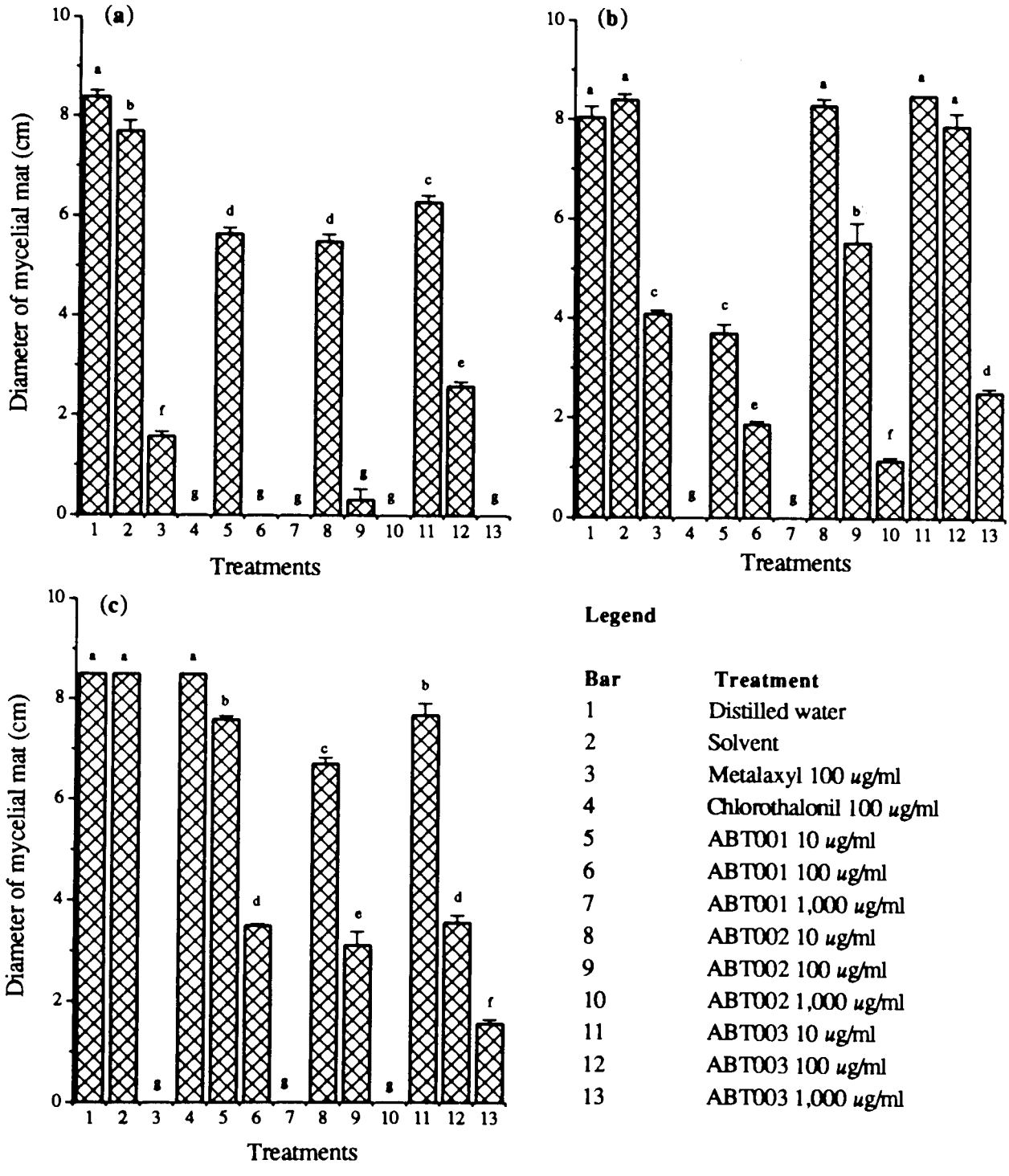


Figure 5: Antimycotic test of different concentrations of ABT001, ABT002, and ABT003 compared with that of metalaxyl and chlorothalonil against three fungi on agar plates as expressed by the diameter of mycelial mat; (a) *Phytophthora infestans*, (b) *Rhizoctonia solani*, (c) *Pythium ultimum*. Bars superscripted with the same letter in each graph are not significantly different ($P < 0.05$).

incubated for 48 h. In plates amended with 0.01 and 0.1 mg/ml of ABT001, radial fungal growth on both plates was significantly slower than that in the control plates with distilled water or solvent, and the rate of growth of *R. solani* on the 0.01 mg/ml ABT001 treatment was not significantly different from that of the metalaxyl treatment. At the end of the experiment, the mycelial mats of *R. solani* on 0.1 mg/ml of ABT001 treatment were less dense than in the control plates.

The highest concentration of ABT001 inhibited growth of *P. ultimum* but when the mycelial plugs were transferred onto untreated PDA, good fungal growth was observed within 48 h. Lower concentrations of ABT001, at 0.01 and 0.1 mg/ml, did not inhibit fungal growth over 48 h of incubation (Figure 5c). Although growth on both plates was significantly slower than the control, the mycelial mats were as thick and dense.

At the same concentrations, ABT001 and ABT002 inhibited growth of *P. infestans* similarly (Figure 5a). However, some plates with 0.1 mg/ml of ABT002 showed traces of mycelial growth around the plugs. When the mycelial plugs in this treatment were transferred onto untreated rye agar, there was good fungal growth from each plug after a further 7 d of incubation. Fungal growth was completely inhibited by the 1.0 mg/ml treatment, and no fungal growth was observed after being transferred onto the untreated rye agar for a further 7 d of incubation. ABT002 did not inhibit the growth of either *R. solani* and *P. ultimum*, except for *P. ultimum* at the highest concentration (Figure 5b, c). However, growth of these two fungi was significantly slower than the controls. Traces of *P. ultimum* growth could be observed on the mycelial plugs in the 1.0 mg/ml ABT002 treatment although no radial growth was visible. When these plugs were transferred onto untreated PDA, good fungal growth was observed after 48 h incubation. Chlorothalonil had no effect on growth *of P. ultimum* whereas metalaxyl completely inhibited the growth of this fungus.

All three concentrations of ABT003, significantly slowed fungal growth of *P. infestans* when compared with the control, but only the highest concentration (1.0 mg/ml) completely inhibited fungal growth (Figure 5a). There was no fungal growth after the mycelial plugs from this treatment (1.0 mg/ml) were transferred to untreated rye agar. The aqueous fraction had no significant effect on the growth of *R. solani* except the highest concentration which slowed fungal growth (Figure 5b). Fungal growth of *P. ultimum* was significantly slowed by the increasing concentrations of ABT003 (Figure 5c). Although there was radial growth on plates with 0.1 and 1.0 mg/ml of ABT003, the mycelial mats *of P. ultimum* were less dense compared with the control, but growth was not inhibited. Treatment with 0.01 mg/ml of ABT003 had the same marginal effect on the fungal growth of *P. ultimum* as that of 0.01 mg/ml of ABT001.

The solvent treatment generally had no significant effect on the growth of any of the three test fungi. Metalaxyl completely inhibited growth of *P. ultimum* but only slowed growth of *P. infestans* and *R. solani* when compared with the control. Chlorothalonil completely inhibited the growth of *P. infestans* and *R. solani* but did not slow the growth of *P. ultimum*.

3.3. Phytotoxicity Test

Before any of the antimycotic substances of *X. bovienii* could be tested for fungal disease management on plants, their phytotoxic properties had to be examined. Five plant species, representing four different families, were chosen for this study. They were bush bean, *Phaseolus vulgaris* cv. Venture-Blue Lake (Fabaceae); winter wheat, *Triticum aestivum* cv. Monopol (Poaceae); tomato, *Lycopersicon lycopersicum* cv. Fantastic (Solanaceae); potato, *Solanum tuberosum*

cv. Norchip (Solanaceae) and cucumber, *Cucumis sativus* cv. Calypso (Cucurbitaceae).

3.3.1. Supernatant

The 96 h *X. bovienii* supernatant was tested for phytotoxicity because there was strong interest in the initial stage of the project to use the lyophilized supernatant as an agent for fungal control. Replicate plants of the following species and age were used in these tests; 3-week-old bush bean and cucumber plants of uniform growth and size in 13 cm diameter plastic pots (bush bean and cucumber plants had two and four fully mature, true leaves, respectively); 2-week-old winter wheat seedlings in 10 cm diameter plastic pots; 4- to 5-week-old tomato plants at the five-leaf-stage in 13 cm diameter plastic pots.

Approximately 1 ml of supernatant at concentrations of 10.0, 20.0, and 40.0 mg/ml was sprayed evenly with an atomizer on the foliage of the four plant species. The desired concentration of supernatant was prepared by dissolving the lyophilized 96 h supernatant (see section 2.2.2.) in distilled water. The 20.0 mg/ml solution represented the approximate concentration of a fresh 96 h culture supernatant. As controls, distilled water and TSB at concentrations of 27.5 and 55.0 mg/ml, were used. After treatment, plants were placed randomly in their respective growing environments (see section 2.6). Cucumber, bush bean and tomato plants were assessed for phytotoxic symptoms on the third day whereas wheat plants were assessed on the fifteenth day after treatment. Phytotoxicity was assessed using a 1-5 scale, as described in section 3.3.4. Growth of the treated plants was compared visually with that of plants treated with distilled water. Each treatment was replicated nine times and this experiment was not repeated.

3.3.2. Organic fraction (ABT001)

The phytotoxic properties of ABT001 were evaluated on bush bean, grass and potato, common plant hosts of the three selected test fungi.

I. Bush bean

Planting material was autoclaved for 1 hour twice, with a 3 d interval between treatments. The autoclaved soil was kept at room temperature for 1 month to release free ammonia and to monitor visually for fungal contamination. Contaminated soil was either discarded or reautoclaved. Seeds were treated with ABT001 at rates of 0.3, 1.5, and 7.5 mg/g of seed. The desired concentration of ABT001 was dissolved in 2 ml of acetone and mixed with diatomaceous earth as carrier in a 250 ml round flask (Pyrex). The acetone was evaporated off under vacuum, leaving behind a layer of yellow, diatomaceous earth on the wall of the flask. A 2 % methyl cellulose solution (w/v), for use as a sticker, was prepared by dissolving methyl cellulose in almost boiling, distilled water and cooling to room temperature. Seeds were mixed with methyl cellulose and added into the flask containing diatomaceous earth with ABT001. Seeds were coated evenly with the mixture by gently rotating the flask by hand, and the seeds were then air-dried overnight on aluminum foil. For each gram of seed, approximately 3 mg diatomaceous earth and 0.7 mg methyl cellulose was used. Control seeds were treated with distilled water, or a mixture of diatomaceous earth and methyl cellulose. There were 25 seeds in each treatment, and eight to nine seeds were planted per row in a soil flat containing sterilized garden mix. Each treatment was replicated four times. Soil flats were arranged in a completely randomized design and maintained for 10 d in a growth room with a 16 h photoperiod at 25 °C. Each treatment was evaluated for the percentage germination, and seedling height on the fifth and tenth day after seeding. A seed was scored as germinated when

both cotyledons were raised above the soil surface. Seedling height was determined by measuring the length of the hypocotyl with hand-held calipers. The percentage seed germination in different treatments was compared using a nonparametric analysis of variance (Friedman's test) followed by Bonferroni's test. Data were analyzed on the assumption that the soil within and between flats was homogenous. This experiment was not repeated.

II. Grass

Five-week-old, potted grass plants were treated with ABT001 at concentrations of 2.0, 10.0 and 20.0 mg/ml. The desired concentration was prepared by dissolving the ABT001 in a solution of Tween 20 (see section 3.2.1.), and to deliver the ABT001 solution to the plant, 2.0 % methyl cellulose (see section 3.3.2.-I) and diatomaceous earth were added at the rate of 10.0 mg/ml (w/v) as sticker and carrier, respectively. As control, plants were treated with distilled water or a mixture of diatomaceous earth and methyl cellulose in a solution of Tween 20. Approximately 1 ml of ABT001 was applied to the grass blades of each pot with an atomizer, after which the pots were covered immediately with transparent, polyethylene bags to maintain high humidity and placed randomly in a Conviron growth chamber with a 16 h photoperiod at 28 °C. Potted grass was assessed for phytotoxic symptoms on the eighth day, using a 1-5 scale, as described in section 3.3.4. Each treatment was replicated eight times, and this experiment was repeated once.

III. Potato

Four to five-week-old potato plants with five mature, compound leaves were used. Approximately 3 ml of ABT001 was applied to the compound leaves of each potato plant at concentrations of 2.0, 10.0 and 50.0 mg/ml as described for

potted grass. The same type of controls were used. Treated plants were covered with polyethylene bags to maintain high humidity and placed in a Conviron growth chamber with a 16 h photoperiod at 20 °C. Plants were assessed for phytotoxic symptoms on the eighth day, using a 1-5 scale, as described in section 3.3.4. Each treatment was replicated five times and the experiment was repeated once.

3.3.3. 'Whole' fraction (ABT002)

The 'whole' fraction was evaluated for phytotoxic properties only on potato plants. Approximately 3 ml of ABT002 was applied to each potato plant using the same procedures, concentrations and controls as described in section 3.3.2.-III. However, in this experiment, distilled water was used as the solvent instead of Tween 20 solution. Each treatment was replicated five times, and this experiment was repeated once.

3.3.4. Rating system for phytotoxicity assessment

A 5-point rating system was used to describe and quantify the phytotoxic damage to treated plants. Since different plant species expressed different phytotoxic symptoms at different times after treatment, a different rating system was developed for each plant species used, as described in Table 7a-d. This phytotoxic assessment was done by visual observation of each treated plant for approximately 1 minute at the end of the experiment. Plants that expressed similar phytotoxic damage were grouped under the same rating system. Only the treated leaves of potted bush bean, cucumber, tomato and potato plants were assessed by the 5-point rating system for phytotoxicity, and the rating was given based on the average damage to the treated leaves of each plant. In potted wheat and grass, ten leaf blades were randomly excised from each replicate treatment,

Table 7a: Details of the 5-point rating system for phytotoxic damage assessment of potted cucumber, bush bean, and tomato plants on the third day after treatment, expressed as a mean rating for all treated leaves on each plant.

Rating	Description of phytotoxic symptoms
1	No symptoms of phytotoxic damage
2	Moderately curled leaf with no chlorotic or necrotic lesions
3	Necrotic streaks along the veins and/or along the margins of almost all treated leaves
4	Yellow, necrotic lesions of various sizes covering ≤ half of leaf area, predominantly at the tip and/or along the margins of almost all treated leaves; terminal bud with outer leaves necrotic
5	Yellow, necrotic lesions of various sizes covering ≥ half of leaf area, predominantly at the tip and/or along the margins of almost all treated leaves; terminal bud with outer leaves necrotic

Table 7b: Details of the 5-point rating system for phytotoxic damage assessment of potted wheat seedlings on the fifteenth day after treatment, expressed as a mean rating for ten leaf blades.

Rating	Description of phytotoxic symptoms
1	No symptoms of phytotoxic damage
2	Brown, necrotic lesions ≤ 1 mm in diameter, covering ≤ 10 % of blade area of almost all treated leaves
3	Brown, necrotic lesions 1-2 mm in diameter, covering ≤ 20 % of blade area of almost all treated leaves
4	Brown, necrotic lesions covering ≤ 50 % of blade area of almost all treated leaves
5	Brown, necrotic lesions covering ≥ 50 % of blade area of almost all treated leaves or dead seedling

Table 7c: Details of the 5-point rating system for phytotoxic damage assessment of potted grass plants on the eighth day after treatment, expressed as a mean rating for ten leaf blades.

Rating	Description of phytotoxic symptoms
1	No symptoms of phytotoxic damage
2	Yellow, necrosis extending ≤ 10 % of blade length from the tip of almost all treated leaves
3	Yellow, necrosis extending 10-20 % of blade length from the tip of almost all treated leaves
4	Yellow, necrosis extending up to 50 % of blade length from the tip of almost all treated leaves
5	Yellow, necrosis extending ≥ 50 % of the blade length from the tip of almost all treated leaves or dead grass

Table 7d: Details of the 5-point rating system for phytotoxic damage assessment of potted potato plants on the eighth day after treatment, expressed as a mean rating for all treated leaves on each plant.

Rating	Description of phytotoxic symptoms
1	No symptoms of phytotoxic damage
2	Necrotic streaks along the edges of almost all treated leaves
3	Necrotic streaks along the veins and/or along the margins of all treated leaves with small, sporadic, chlorotic lesions
4	Yellow, necrotic lesions covering ≤ half of leaf area, predominantly at the tip and/or along the margins of almost all treated leaves; terminal bud with outer leaves necrotic
5	Yellow, necrotic lesions covering ≥ half of leaf area, predominantly at the tip and/or along the margins of almost all treated leaves; terminal bud with outer leaves necrotic

and the rating was based on the average phytotoxic symptoms shown by these leaf blades for each treatment. The average rating for each treatment was compared using a nonparametric analysis of variance (Friedman's test) followed by Bonferroni's test. Plants were maintained for a further 7 d after they had been assessed in order to monitor and describe overall growth of the treated and control plants visually.

3.3.5. Results

I. Supernatant

Phytotoxic damage was observed on the treated foliage of bush bean plants 3 d after treatment with different concentrations of supernatant (Figure 6a). Plants treated with 10.0 mg/ml of supernatant had moderately curled cotyledons and true leaves, with necrotic streaks at the tip and margins of the leaf where supernatant droplets accumulated. Necrotic streaks were observed also along the veins. There was no significant difference in the degree of phytotoxic damage between plants treated with 10.0 mg/ml of supernatant and 27.5 mg/ml of TSB or between plants treated with 20.0 and 40.0 mg/ml of supernatant and 55.0 mg/ml TSB. However, there was a significant difference in the degree of phytotoxic damage between plants treated with different concentrations of supernatant, and this damage was proportional to the concentration. Generally, the supernatant and TSB treatments expressed similar levels of phytotoxicity, but these treatments did not appear to slow plant growth over 10 d except at the highest concentration, compared with the water control. The highest concentration of supernatant and TSB resulted in wrinkled leaves and in terminal buds with necrotic leaves.

Three-week-old cucumber plants were more sensitive to the higher concentrations of lyophilized supernatant and TSB than were the three other species of plants tested (Figure 6b). All treated plants showed significant

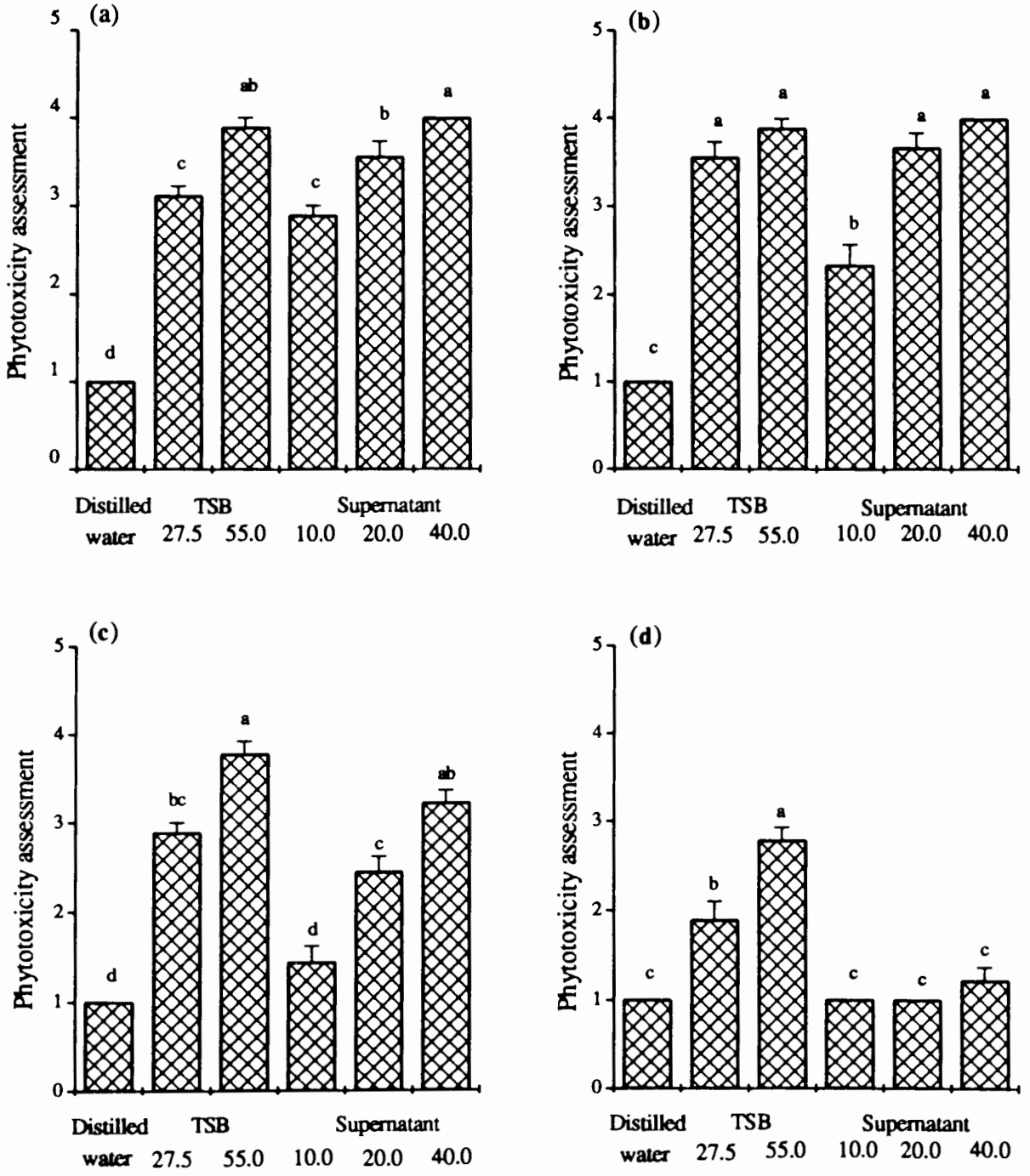


Figure 6: Phytotoxicity assessment of (a) bush bean, (b) cucumber, (c) tomato and (d) wheat with a 5-point rating system after treatment with one of three concentrations of lyophilized supernatant (mg/ml), two of TSB (mg/ml) or distilled water. Bars superscripted with the same letter(s) in each graph are not significantly different ($P < 0.05$).

phytotoxic damage as compared with the water control. There was no significant difference in phytotoxic damage between plants treated with 20.0 and 40.0 mg/ml of lyophilized supernatant and 27.5 and 55.0 mg/ml of TSB. The majority of these treated plants had moderately curled leaves with necrotic lesions on both cotyledons and true leaves, and on the leaves of the terminal bud. When these plants were observed over 10 d, elongated internodes with undeveloped or abnormally shaped leaves were formed from the damaged terminal buds. Plants treated with 10.0 mg/ml of lyophilized supernatant expressed less severe symptoms, with moderately curled leaves and/or minor necrotic streaks on the tip or along the margins of the leaf where the treatment droplets accumulated. When observed over 10 d, this treatment had no negative effect on plant growth when compared with the water control.

On tomato plants, the level of phytotoxic damage increased proportionally with the concentration of lyophilized supernatant (Figure 6c). Although some of the tomato plants treated with 10.0 mg/ml of lyophilized supernatant had curled leaflets, this damage was not significantly different from that observed on tomato plants treated with distilled water. Moreover, this treatment had no negative effect on plant growth when observed over a 10 d period. The phytotoxic damage on tomato plants treated with 40.0 mg/ml of supernatant was similar to that observed in plants treated with either 27.5 or 55.0 mg/ml of TSB, in the yellow necrotic lesions on some treated leaves, and damaged terminal bud on some replicates. When these plants were observed over a 10 d period the leaves with yellow necrotic lesions turned chlorotic. Similar symptoms were observed on tomato plants with 20.0 mg/ml treatment, but were less severe with no chlorotic leaves or damage terminal buds.

Two-week-old wheat seedlings did not express any visual signs of phytotoxicity at all three concentrations of lyophilized supernatant (Figure 6d).

However, when wheat seedlings were treated with TSB, necrotic lesions were observed at both treatment levels when these seedlings were assessed on the fifteenth day after application. The TSB treatments exhibited significantly more phytotoxic damage than any of the treatments with the supernatant. However, none of the treatments had a negative effect on plant growth.

II. Organic fraction

A. Bush bean

The organic fraction, ABT001, did not significantly affect germination of bush bean seeds when assessed on day 5 or day 10 except at the highest level (7.5 mg/g of seed) where germination was temporarily slowed on day 5 (Figure 7). The highest concentration of ABT001 also significantly slowed hypocotyl elongation when compared with all other treatments, and this trend persisted to day 10 (Figure 8). When the seedlings in the 7.5 mg/g of seed treatment were examined at the end of the experiment, almost all germinated seedlings appeared stunted, and the hypocotyls were either very short (ca. 3.65 cm) (Appendix III) or coiled with irregular patterns. The carrier had no significant effect on bush bean germination or seedling growth.

B. Potted grass

The phytotoxic damage increased proportionally with increasing concentrations of ABT001 (Figure 9), and showed an increase in yellow necrosis extending from the tip of the blade. Grass treated with 2.0 mg/ml of ABT001 had yellow necrosis on only old leaf blades and on the tip of the younger blades. This symptom was similar in the 20.0 mg/ml treatment, but much more severe. Except for the newly developed blades, almost all the treated blades turned chlorotic over

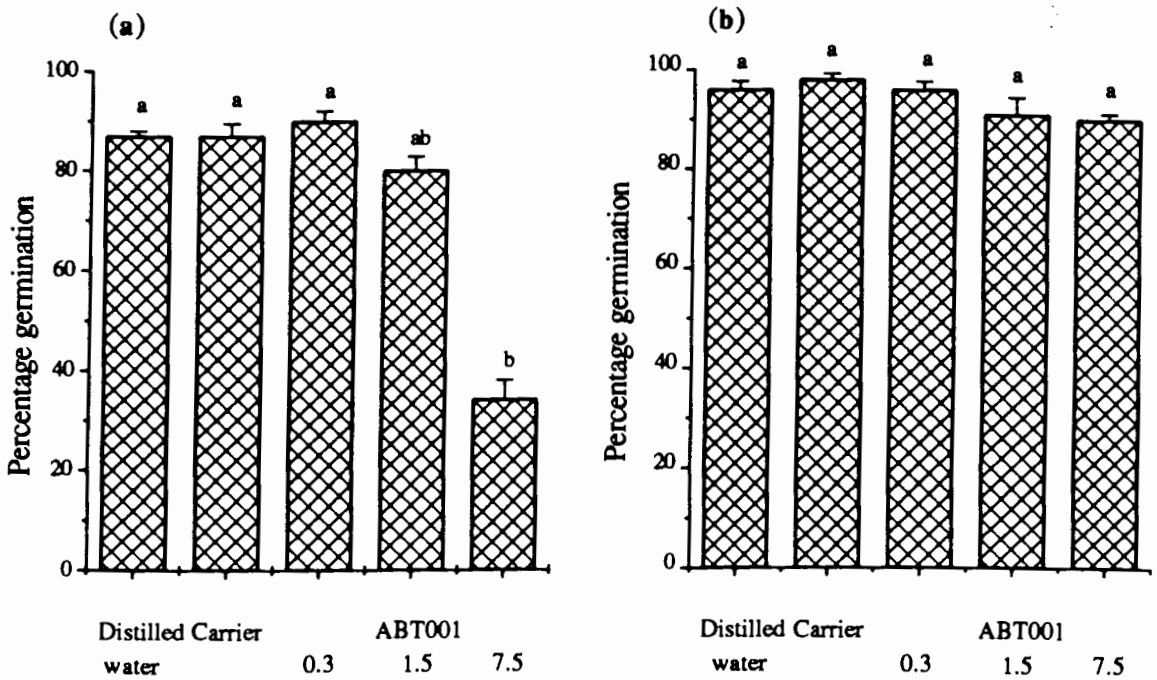


Figure 7: Phytotoxicity effects of three concentrations of ABT001 (mg/g of seed), as a seed dressing, on the germination of bush bean seeds, assessed on (a) day 5 and (b) day 10 after seeding. Bars superscripted with the same letter(s) in each graph are not significantly different ($P < 0.05$).

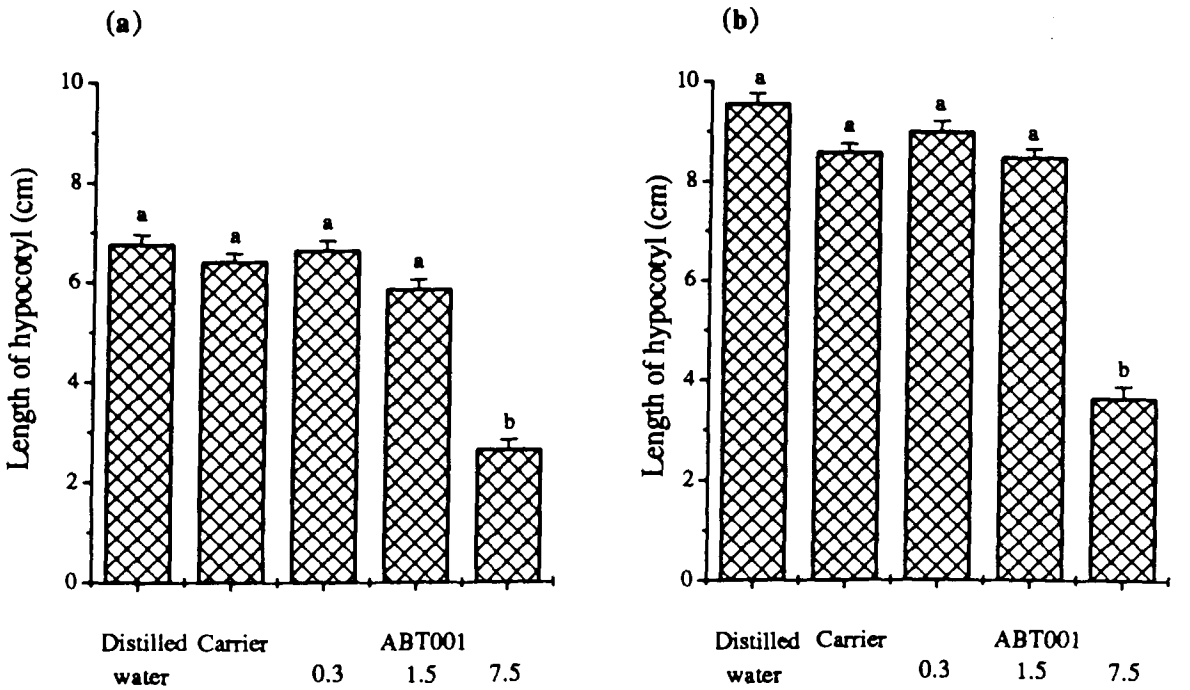


Figure 8: Phytotoxicity effects of three concentrations of ABT001 (mg/g of seed), as a seed dressing, on hypocotyl length of bush bean seedlings, assessed on (a) day 5 and (b) day 10 after seeding. Bars superscripted with the same letter in each graph are not significantly different ($P < 0.05$)

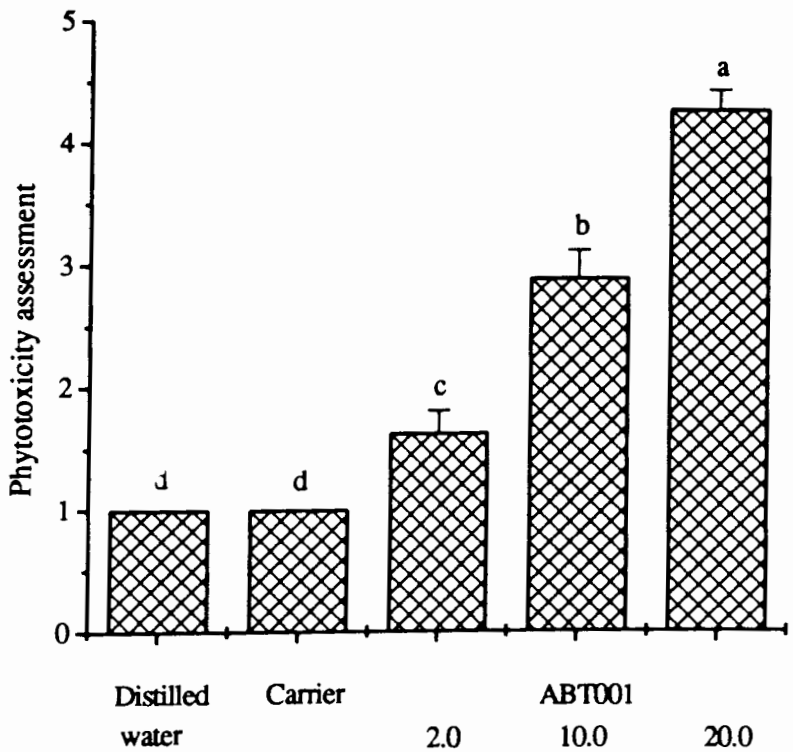


Figure 9: Phytotoxicity assessment of 5-week-old potted grass, using a 5-point rating system, 8 d after treatment with three concentrations of ABT001 (mg/ml). Bars superscripted with the same letter are not significantly different ($P < 0.05$).

8 d following this treatment. None of these symptoms was present in the control plants, either those treated with distilled water or with the carrier.

C. Potato

Phytotoxic symptoms on potato plants were visible as early as 24 h following treatment with 50 mg/ml of ABT001. The leaves of these plants showed characteristic symptoms of epinasty, the downward curling or bending of the leaflets (Figure 10). There was no sign of wilting but rather the leaflets appeared slightly turgid. These symptoms were not visible in the control potato plants but were present, though to a lesser degree, in plants treated with 10.0 mg/ml of ABT001.

When the potato plants were assessed with the 5-point rating system on day 8, the above symptoms had disappeared. ABT001 was phytotoxic at all levels of treatment and increased significantly with increasing concentrations (Figure 11a). The phytotoxic effects were observed as an increasing number and size of yellow necrotic lesions on the treated leaflet, predominantly around the tip and margins of the leaflet as the concentration of the treatment increased. Treatment with 50.0 mg/ml of ABT001 resulted in some of the replicate plants with damaged terminal buds where young and undeveloped leaves turned necrotic. In these plants, the damage was permanent. Plants treated with 10.0 mg/ml had necrotic streaks along the veins and margins of the treated leaflets and chlorotic lesions on the leaflets where the treatment droplets accumulated. Plants treated with 2.0 mg/ml had only necrotic streaks at the tip of the leaflets, and otherwise the leaflets appeared as healthy as those in the water control. In both treatments, at 2.0 and 10.0 mg/ml of ABT001, there was no damage to the terminal buds. Other than those plants that had shown damaged terminal buds, none of the ABT001 treatments caused signs of phytotoxicity on the new compound leaves that

Figure 10: Symptoms of epinasty on 4- to 5-week-old, potted potato plant, 24 h after treatment with 50 mg/ml of ABT001, compared with that of potato plant treated with distilled water (untreated).

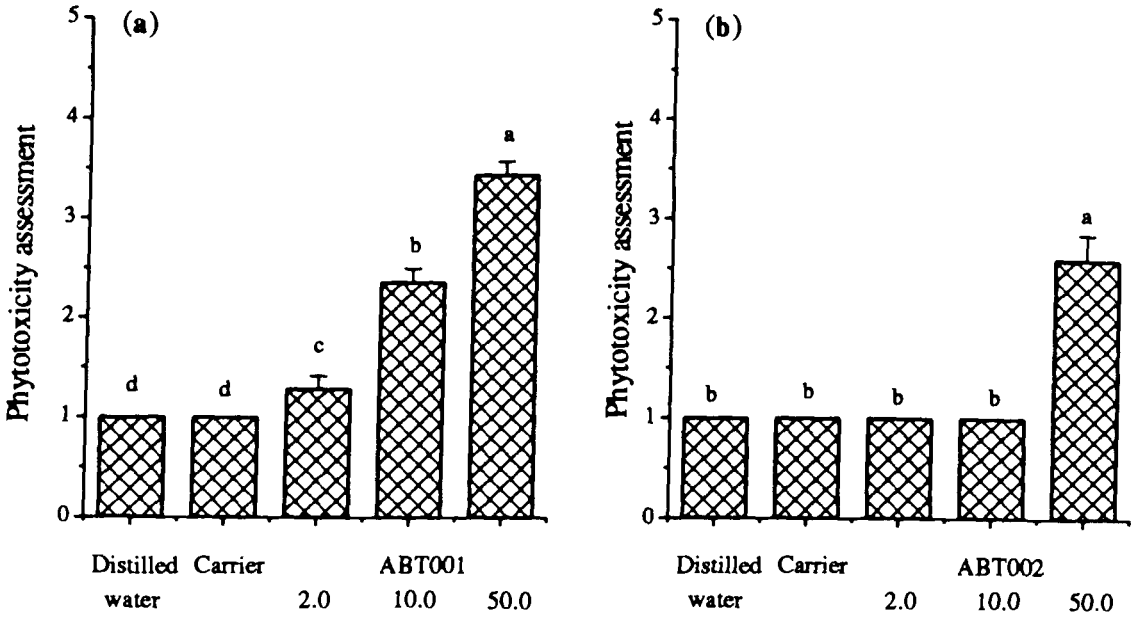


Figure 11: Phytotoxicity assessment of 4- to 5-week-old potted potato plants, using a 5-point rating system, 8 d after treatment with three concentrations (mg/ml) of either (a) ABT001 or (b) ABT002. Bars superscripted with the same letter in each graph are not significantly different ($P < 0.05$).

developed after treatment. The carrier had no phytotoxic effect on the potato plants.

III. 'Whole' fraction

A. Potato.

Phytotoxic symptoms were observed on potato plants as early as 24 h following treatment with ABT002 at 50.0 mg/ml. These plants showed the characteristic symptoms of epinasty with no chlorotic or necrotic lesions. These symptoms were not observed in other treatments. When the same plants were assessed for phytotoxic damage on day 8 with a 5-point system, the above symptoms had disappeared. Phytotoxic damage was observed only on plants treated with the highest concentration of ABT002 (Figure 11b). These plants had necrotic lesions along the veins and margins of the leaflets and chlorotic lesions on the leaflet where the treatment droplets accumulated. However, there was no measurable effect on plant growth. Plants treated with the carrier showed no phytotoxicity and were comparable with those in the water treatment. New compound leaves that developed after treatment were healthy and showed no sign of phytotoxicity.

3.4. Efficacy of the Antimycotic Substances against Fungi on Potted Plants

3.4.1. ABT001 against *P. ultimum* on bush bean.

Pythium ultimum was artificially incorporated into sterilized garden mix at the rate of 1,000 cfu/g of soil in 53 x 27 cm plastic flats that contained approximately 2 kg of soil. Seeds were treated with ABT001 at the same rates, and with the same controls as described in section 3.3.2.-I. The same

experimental procedure was also followed. For comparison, Apron TL (11.5 % metalaxyl a.i.), was coated on the seeds at the rate of 1.5 mg a.i. (13 mg Apron TL) per gram of seed with methyl cellulose (0.7 mg). There were 25 seeds in each treatment, and each treatment was replicated four times. Soil flats were arranged in a complete randomized block design, and maintained for 10 d in a growth room with a 16 h photoperiod at 25 °C. Each treatment was evaluated for the percentage of seed germination on the fifth and tenth day and for the percentage of seedlings with healthy shoot and root systems on the tenth day. Germinated seedlings with no black or brown lesions on the hypocotyl stem and no sign of wilting were regarded as healthy seedlings. Root systems with no brown root lesions or decaying root hairs were regarded as healthy. The percentages of germinated seedlings were compared using a nonparametric analysis of variance (Friedman's test) followed by the Bonferroni's test. Data were analyzed assuming that the soil in all flats was homogenous and that adjacent flats did not influence each other. This experiment was not repeated.

To confirm that the diseased seedlings were infected by *P. ultimum* and not by other fungal species, three pieces of tissue (ca. 4 mm^2) were cut with a sterile scalpel from the edges of hypocotyl lesions from five arbitrarily selected diseased seedlings or from ungerminated seeds in each replicate, surface sterilized in 0.5 % sodium hypochlorite (v/v) for 3 minutes, rinsed in sterilized distilled water for 3 minutes, and plated on Pythium-selective medium [P_5ARP(H)] (Appendix VII) (Jeffers and Martin, 1986). Plates were incubated at 20 °C and examined periodically for fungal colony development. Subcultures were made onto PDA and maintained at 20 °C. The plated fungal cultures were compared morphologically under a light microscope with a stock culture of *P. ultimum*.

3.4.2. ABT001 against *R. solani* on potted grass.

The method used was modified from that of Sanders and Cole (1986). Five-week-old potted grass was treated with ABT001 at rates of 2.0, 10.0, and 20.0 mg/ml. Desired concentrations of ABT001 were prepared and delivered to the potted grass as described in section 3.3.2.-II. The same type of controls were established. For comparison, Bravo 720 (54 % chlorothalonil a.i.) was used at the rate of 10.0 mg/ml a.i. (18.6 mg/ml Bravo 720). Approximately 1 ml of treatment solution was sprayed on each pot of grass with an atomizer. After 24 h of incubation in a Conviron growth chamber at 28 °C with a 16 h photoperiod, potted grass was infected with *R. solani* by placing 10 kernels of rye inoculum in the center of the pot. After inoculation, pots were covered with transparent, polyethylene bags to maintain high humidity, and replaced randomly in the growth chamber with the same environmental conditions for optimum disease development. The polyethylene covers were removed from the pots, and the protective efficacy of ABT001 was evaluated on the seventh day after inoculation. This timing was determined by the rate of spread of the fungus in the control pots. The experiment was terminated when the grass in the control pots was completely infected. At this time, all inoculated pots were evaluated individually for disease severity. A 0-10 visual rating scale was used such that each point on the scale corresponded to the diameter (cm) of the infected area of the potted grass: 0 = no disease, 1 = 1 cm of infected grass, 2 = 2 cm of infected grass, through to 10 = all grass foliage infected. The average diameter of the area covered by the fungus infected grass in each treatment was compared using a nonparametric analysis of variance (Friedman's test) followed by the Bonferroni's test. This experiment was repeated once.

To confirm the that the diseased grass was infected by *R. solani* and not by other fungi, three pieces of infected grass leaf (ca. 4 mm^2) were cut with sterile

scissors from each of five arbitrarily selected pots in each treatment, surface sterilized in 0.5 % sodium hypochlorite (v/v) for 3 minutes, rinsed in sterilized distilled water for 3 minutes, and plated on 0.39 % PDA amended with 0.005 % chlorotetracycline hydrochloride (w/v) and 0.005 % streptomycin sulfate (w/v). After 48 h of incubation at 24 °C, plates were examined for fungal colony development. Subcultures were made onto 3.9 % PDA, maintained at 24 °C, and the plated fungal cultures were then compared morphologically under a light microscope with the stock culture of *R. solani*.

3.4.3. ABT001 against *P. infestans* on potato plants

I. *Phytophthora infestans* on detached potato leaflets

Four to five-week-old, potted potato plants of about the same size and with five mature leaves were randomly selected and divided into groups of three. Each group was randomly treated with one of the concentrations of ABT001 or the controls, as described in section 3.3.2.-III, and similar procedures as described in this section were used to prepare and deliver the desired concentration of ABT001 to a potted potato plant. For comparison, Bravo 720 was applied to the potato plants at rate of 10.0 mg/ml a.i. (18.6 mg/ml Bravo 720). Approximately 3 ml of treatment solution was sprayed evenly on each plant with an atomizer. Treated plants were randomly placed in a growth chamber with a 16 h photoperiod, at 16-20 °C. After 24 h the plants were removed from the growth chamber and immediately sprayed with a fine mist of distilled water. Randomly, one leaflet was aseptically detached with a scalpel from each of the three potted potato plants from the same treatment. All the three leaflets of various sizes were placed in the same Petri plate containing 15 ml of solidified, 1.5 % Anachemia water agar. One 0.5 cm diameter agar plug from a 2-week-old culture of *P. infestans* was placed, mycelial face down on the abaxial surface of each moist leaflet at the junction of

the mid vein and a lateral vein. The Petri plates were then sealed with Parafilm and placed randomly in an incubator, in darkness at 16 °C. After 48 h, the inoculum plug was removed, the dish resealed and returned to the chamber. On the seventh day after inoculation, leaflets were assessed qualitatively for the presence of blight lesions under a dissecting light microscope. A successful infection showed as a dark lesion, on both the ventral and dorsal surface of the leaf, covered by thick, growing *P. infestans* hyphae with characteristic sporangia. The average percentage of the inoculated leaflet infected with *P. infestans* in each treatment was compared using a nonparametric analysis of variance (Friedman's test) followed by Bonferroni's test. Each treatment was replicated five times and this experiment was repeated once.

II. *Phytophthora infestans* on potted potato plants

Four to five-week-old, potted potato plants of about the same size and each with five mature leaves were randomly selected and divided into groups of five. Each group was randomly treated with two concentrations of ABT001 at 5.0 and 10.0 mg/ml, or with the control spray as described in section 3.3.2.-III. ABT001 at 50.0 mg/ml was not tested due to its severe phytotoxic effects. The same procedures as described in this section were used to prepared and deliver the desired concentration of ABT001 to the potted potato plant. For comparison, Bravo 720 was used at the rate of 10.0 mg/ml a.i. (18.6 mg/ml Bravo 720). Approximately 3 ml of treatment solution was sprayed evenly on each plant with an atomizer. Treated plants were randomly placed in a growth chamber with a 16 h photoperiod, at 16-20 °C. After 24 h the plants were removed from the growth chamber and immediately sprayed with a fine mist of water to keep the surface of the leaflets moist. The terminal leaflet of the five mature compound leaves of the potato plants were inoculated with *P. infestans* spores by gently

rubbing the mycelial surface of a 0.5 cm diameter agar plug from 2-week-old cultures on the abaxial surface of a moist terminal leaflet, at the junction of the mid vein and a lateral vein. Subsequently, the inoculated plant was covered with a transparent, polyethylene bag to maintain high humidity and replaced randomly into the growth chamber. The plants were kept in the dark for the first 24 h following fungal inoculation, and were sprayed again with a fine mist of water after 48 h of incubation. Disease development was recorded qualitatively on the seventh day after inoculation by assessing the inoculated terminal leaflets for blight lesions under a dissecting light microscope (see section 3.4.3-I). The average percentage of the inoculated terminal leaflet infected with *P. infestans* in each treatment was compared using a nonparametric analysis of variance (Friedman's test) followed by the Bonferroni's test. Each treatment was replicated five times, and this experiment was repeated once.

To confirm that the blighted potato leaflets were infected by *P. infestans* and not by other fungi, three pieces of blighted leaf tissue (ca. 4 mm^2) were cut with sterile scissors from each potted potato plant, surface sterilized in 0.5 % sodium hypochlorite (v/v) for 3 minutes, rinsed in sterilized distilled water for 3 minutes, and plated on rye agar amended with 0.01 % vancomycin (w/v), 0.0025 % nystatin (w/v), and 0.002 % rifampicin (w/v). After 7 d of incubation at 16 °C, plates were examined for fungal colony development. Subcultures were made onto rye agar and maintained at 16 °C. The plated fungal cultures were compared morphologically under a light microscope with the stock culture of *P. infestans*.

3.4.4. ABT002 against *P. infestans* on potato plants

The same procedures as described in section 3.4.3-I and II were used to evaluate the protective effect of the ABT002 against *P. infestans* on potato plants.

However, in these experiments distilled water was used as solvent instead of Tween 20 solution. In the potted potato plant test, ABT002 was tested only at 10.0 and 50.0 mg/ml. Each treatment was replicated five times, and the experiment was repeated once.

3.4.5. Results

I. ABT001 against *P. ultimum* on bush bean

On day 5 after seeding, there was no significant protection of bush bean seeds against *P. ultimum* as shown by the fact that there were no significant differences in percentage of germination between seeds treated with different concentrations of ABT001 and the controls (Figure 12a). Seeds treated with ABT001 at the rate of 7.5 mg/g of seed had a significantly lower percentage of germination, with only 7.0 %, compared with 49.0 and 59.0 % in seeds treated with ABT001 at 0.3 and 1.5 mg/g of seed, respectively (see Appendix IV). The commercial fungicide, metalaxyl, coated on the seeds provided good protection against damping-off and resulted in a 90.0 % rate of germination.

On day 10, a similar trend was observed as that for day 5, but with a higher percentage of germination overall (Figure 12b). There were no significant differences among all treatments except that a significantly higher percentage of seeds treated with metalaxyl germinated. Seeds with the highest concentration of ABT001 had the lowest percentage of germination, with only 38.0 %, followed closely by the controls, 44.0 and 47.0 % for seeds treated with the carrier and distilled water, respectively whereas the percentage germination of seeds treated with metalaxyl was 96 % (see Appendix IV). When the germinated seedlings were assessed for healthy shoots 10 d after treatment, a similar trend to that of the percentage of germination was observed (Figure 13a) However, when the root systems were examined at the end of the experiment all treatments, except the

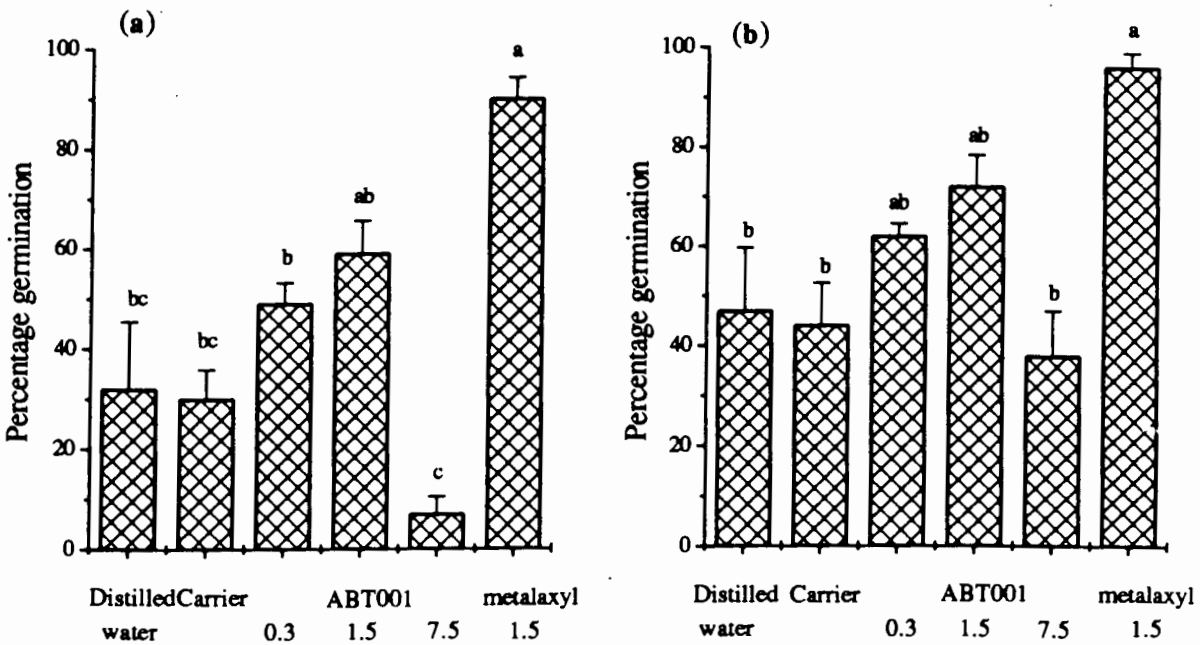


Figure 12: Efficacy of three concentrations of ABT001 (mg/g of seed) or metalaxyl (mg/g of seed), as a seed dressing, on the germination of bush bean seeds in soil inoculated with *Pythium ultimum*, assessed on (a) day 5 and (b) day 10 after seeding. Bars superscripted with the same letter(s) in each group are not significantly different ($P < 0.05$).

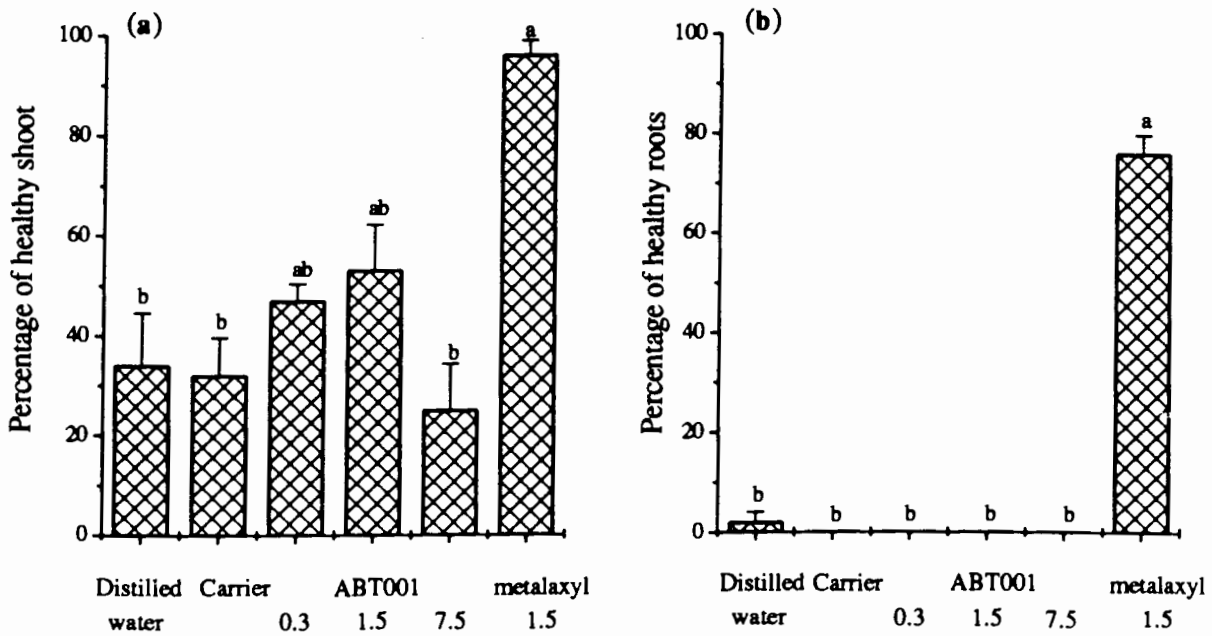


Figure 13: Efficacy of three concentrations of ABT001 (mg/g of seed) or metalaxyl (mg/g of seed), as a seed dressing, on the health of the (a) shoot and (b) root systems of bush bean seedlings in soil inoculated with *Pythium ultimum*, assessed on day 10 after seeding. Bars superscripted with the same letter(s) in each group are not significantly different ($P < 0.05$).

metalaxyl, showed brown lesions and severely damaged root hairs, symptoms of *P. ultimum* infection (Figure 13b). When the ungerminated seeds from the 7.5 mg/g ABT001 treatment were recovered from the soil and examined, most of the seeds were severely infected with *P. ultimum*. Up to 76.0 % of the seedlings from seeds treated with metalaxyl had no sign of infection in their root systems and the remainder of the seedlings had only minor lesions and continued to maintain a vigorous root system. The fungal hyphae growing in ungerminated seeds and infected seedlings recovered from infested soil flats were almost exclusively *P. ultimum* when subcultured on selective media.

II. ABT001 against *R. solani* on potted grass

None of the ABT001 treatments significantly decreased fungal infection of the grass and there was no significant difference between treatments (Figure 14). After 7 d of incubation, the fungal mycelia had reached the edge of the pot in most treatments, and diseased grass was evident in the control pots and the potted grass treated with 2.0 and 10.0 mg/ml ABT001. Most of the infection was concentrated at the lower part of the grass foliage, 2-3 cm above the soil surface. The highest concentration of ABT001, 20.0 mg/ml, did not inhibit mycelial growth of *R. solani* whereas treatment with 10.0 mg/ml of chlorothalonil completely inhibited mycelial growth of *R. solani*.

III. ABT001 and ABT002 against *P. infestans* on detached potato leaflets

The efficacies of both the organic and 'whole' fractions were initially assessed qualitatively as protectant sprays against *P. infestans* on detached potato leaflets. This experiment was conducted to determine the lowest concentrations of ABT001 and ABT002 that should be used on potted potato plants to control late blight with the least phytotoxicity.

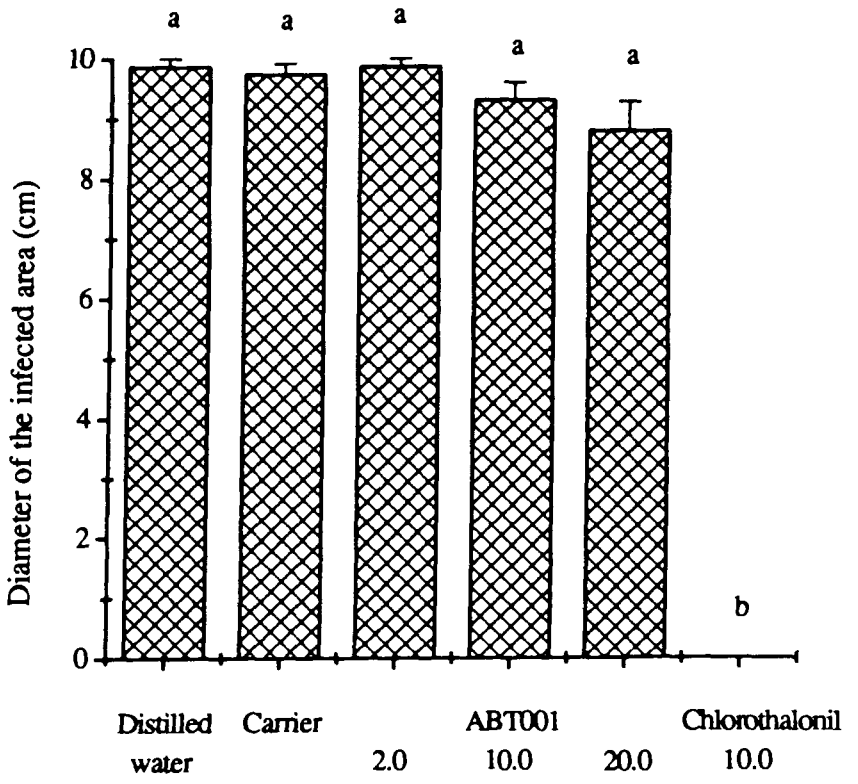


Figure 14: Diameter of the grass area infected with *Rhizoctonia solani*, 8 d after treatment with one of three concentrations of ABT001 (mg/ml) or with chlorothalonil (mg/ml). Bars superscripted with the same letter are not significantly different ($P < 0.05$).

ABT001 at both 10.0 and 50.0 mg/ml inhibited late blight infection, as there was no sign of white mycelium or black lesions on the surface of the inoculated area of the leaflets when they were examined under a dissecting light microscope 7 d after inoculation (Figure 15 and 16a). This observation was comparable with that of the potato leaflets treated with 10.0 mg/ml of chlorothalonil. However, leaflets treated with 50.0 mg/ml of ABT001 developed phytotoxic symptoms by the fourth or fifth day after treatment with the presence of necrotic lesions along the veins and on the margins of the leaflets where the treatment droplets accumulated. Leaflets treated with 10.0 mg/ml of ABT001 and chlorothalonil developed similar symptoms on day 5 or 6, but to a lesser degree. Treatment with 2.0 mg/ml of ABT001 provided only partial inhibition of late blight infection with 33.3 % of the treated leaflets infected with *P. infestans* (see Appendix V). However, even this low level of treatment significantly decreased the percentage fungal infection of the inoculated leaflets as compared with the controls, and the fungal infection sites were smaller and restricted to the inoculated area. Leaflets with unsuccessful infection had only pinhead, black lesions within the inoculated site with no mycelium present. Infected leaflets treated with distilled water or the carrier alone were fully blighted and had *P. infestans* mycelium with sporangia covering the leaflet surface.

A similar trend was observed on leaflets treated with the ABT002, where the 10.0 and 50.0 mg/ml treatments provided almost complete inhibition of zoospore or sporangium germination and mycelial development. This was statistically comparable with the protection provided by chlorothalonil (Figure 16b). There were no lesions on the inoculated leaflets treated with either 50.0 mg/ml of ABT002 or chlorothalonil. However, 4.2 % of the leaflets treated with 10.0 mg/ml of ABT002 were infected (see Appendix V) and the blighted lesion was limited to the inoculated site. The lower concentration of ABT002

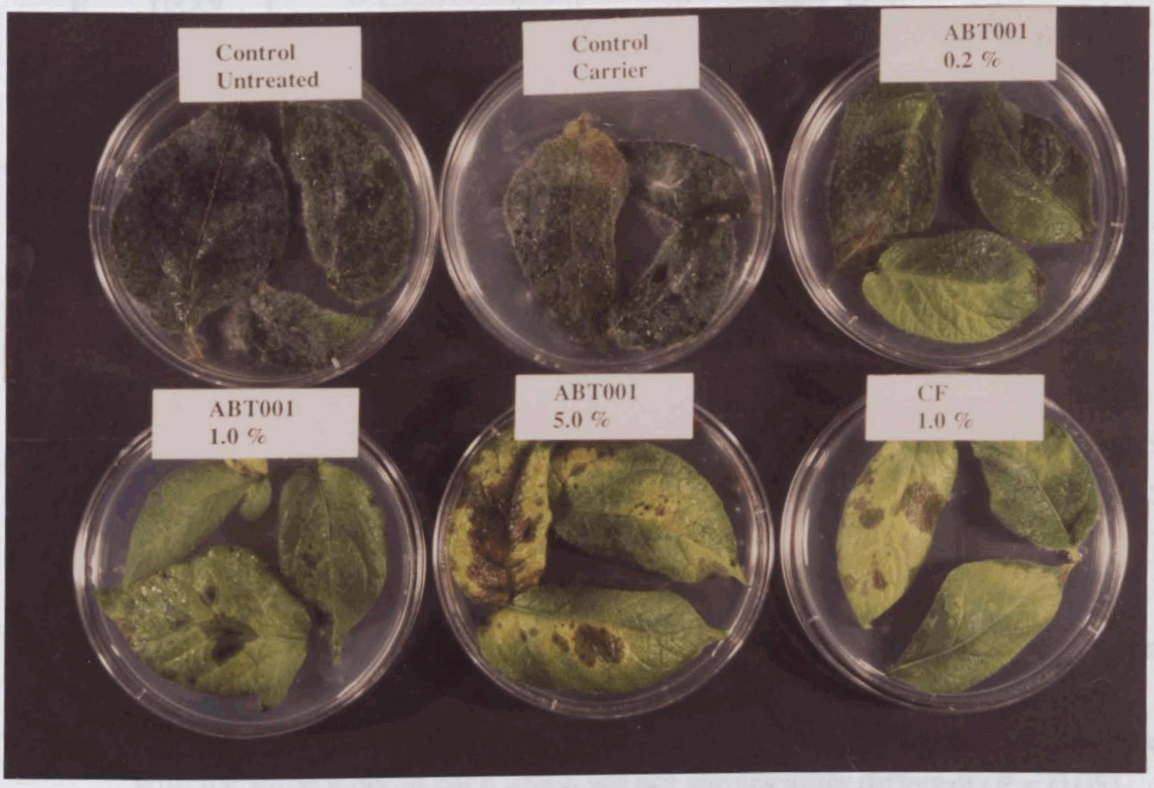


Figure 15: Control of late blight (*Phytophthora infestans*) infection on detached potato leaflets, 8 d after treatment with one of three concentrations of ABT001, 2.0 mg/ml (0.2 %), 10.0 mg/ml (1.0 %) and 50.0 mg/ml (5.0 %), compared with that of distilled water (untreated), carrier and 10.0 mg/ml chlorothalonil (1.0 % CF).

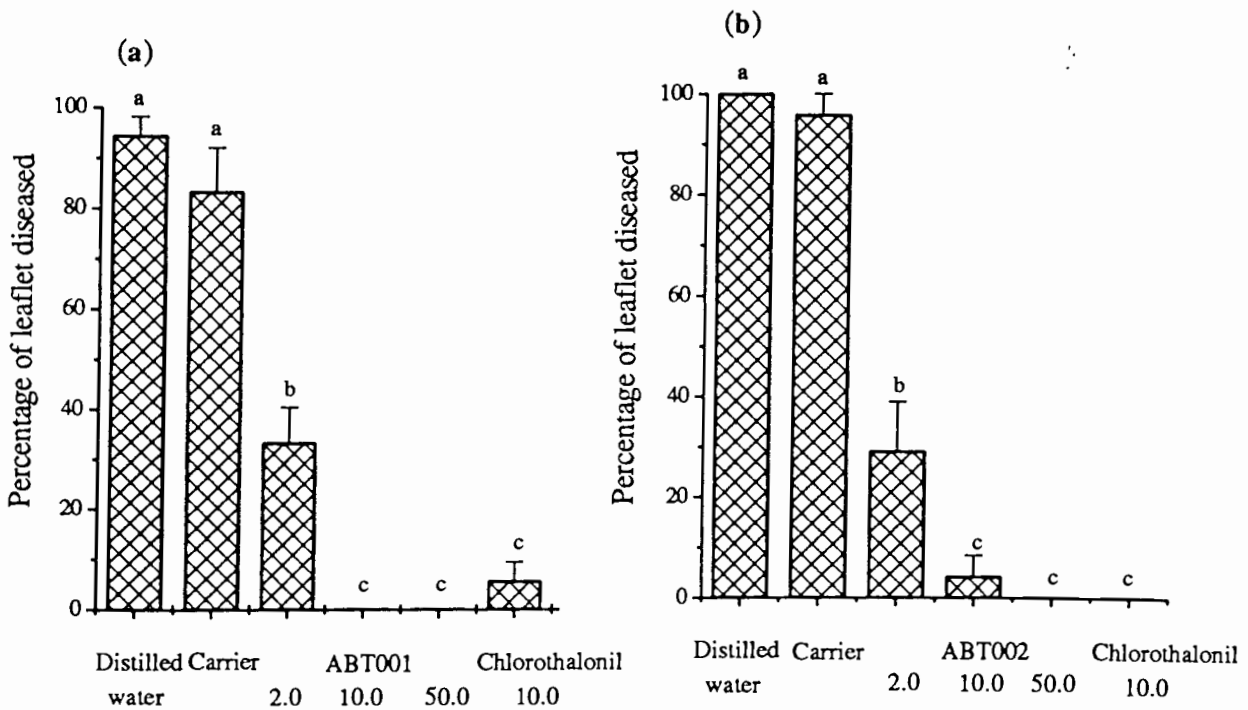


Figure 16: Percentage of detached potato leaflets infected with *Phytophthora infestans* in Petri plates 8 d after treatment with one of three concentrations (mg/ml) of (a) ABT001 or (b) ABT002, or with chlorothalonil. Bars superscripted with the same letter in each graph are not significantly different ($P < 0.05$).

(2.0 mg/ml) significantly decreased late blight infection when compared with the controls, as up to 70.9 % of the treated leaflets were not infected. ABT002 treated leaflets with successful infections had small localized lesions around the inoculated site. The infection proceeded at a slower rate in these leaflets than in the controls. Leaflets treated with distilled water and the carrier had 100 and 95.8 %, respectively, of inoculated leaflets infected, and the leaflets were covered with fungal hyphae.

IV. ABT001 against *P. infestans* on potted potato plants

Treatment at 50.0 mg/ml was not tested as it had been shown to be significantly phytotoxic to the potato leaflets. Due to the condensation that occurred in the polyethylene bags and the continuous growth of the potato plants, it was extremely difficult to assess the rate of infection daily without touching the bags, which could result in the possibility of the inoculum being removed. Therefore, treated plants were examined qualitatively only on day 8, the end of the experiment.

ABT001 provided good control of *P. infestans* on potted potato plants (Figure 17). Only 4.0 % of the inoculated terminal leaflets of potted potato plants treated with 10.0 mg/ml of ABT001 were infected with *P. infestans*, and this was statistically comparable with that of the potato plants treated with 10.0 mg/ml of chlorothalonil (Figure 18a and Appendix VI). There were no signs of white mycelium or black lesions within the inoculated site of the uninfected leaflets. Potted potato plants treated with 5.0 mg/ml of ABT001 had 24.0 % of the inoculated terminal leaflets infected with *P. infestans*. This percentage of infection was significantly lower than both control treatments which were 100 % infected. Infected leaflets treated with 5.0 mg/ml of ABT001 had smaller, localized, blighted lesions when compared with those of the controls. In some of

Figure 17: Control of late blight (*Phytophthora infestans*) infection on 4- to 5-week-old, potted potato plants, 8 d after treatment with (a) 5.0 mg/ml (0.5 %) and (b) 10.0 mg/ml (1.0 %) ABT001, compared with that of distilled water (untreated) and carrier.


a
UNTREATED
CARRIER FOR ABT001
ORGANIC FRACTION
b
UNTREATED
CARRIER FOR ABT001
ORGANIC FRACTION

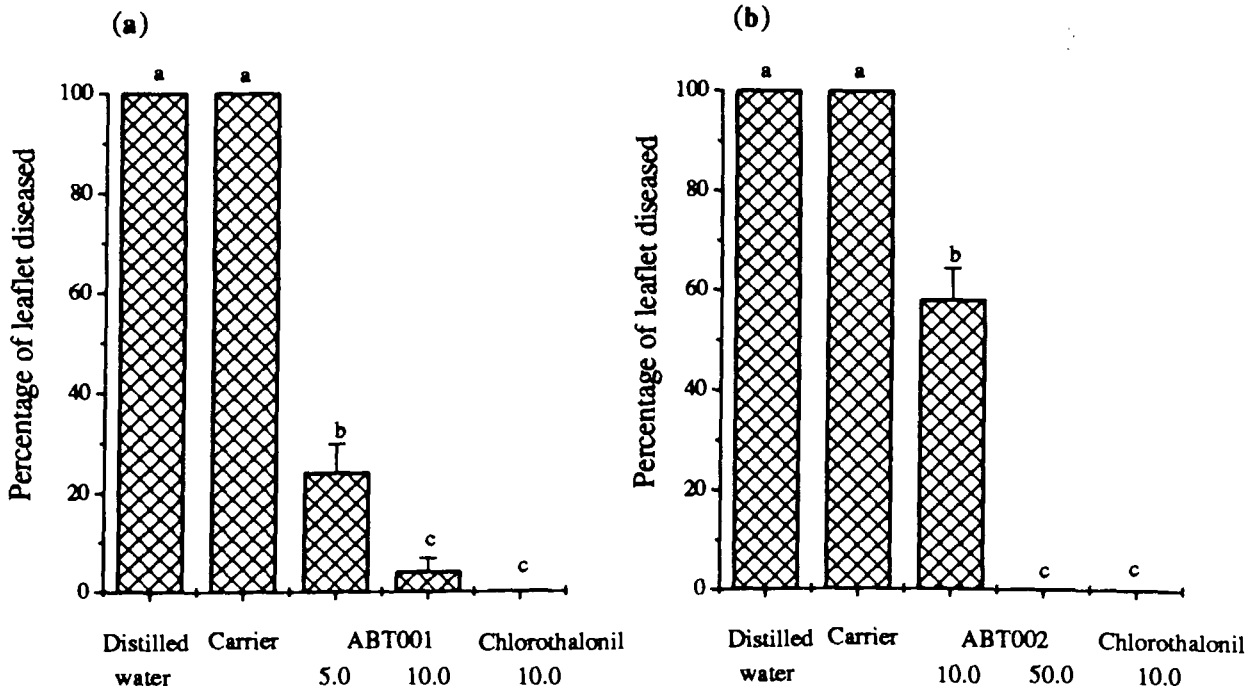


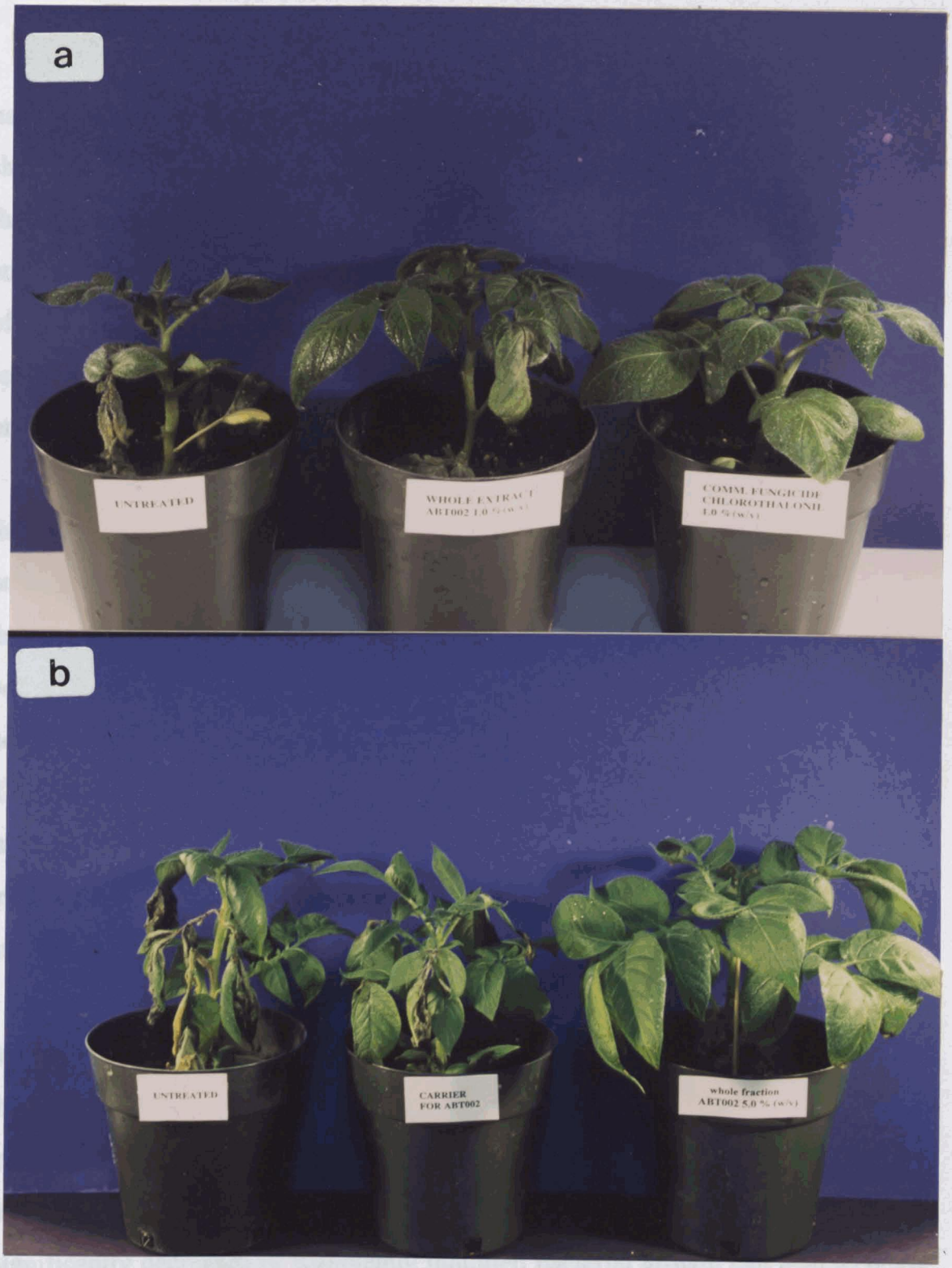
Figure 18: Percentage of inoculated leaflet on 4- to 5-week-old potted potato plant infected with *Phytophthora infestans* 8 d after treatment with one of the two concentrations (mg/ml) of (a) ABT001 or (b) ABT002, or with chlorothalonil. Bars superscripted with the same letter in each graph are not significantly different ($P < 0.05$).

the control plants, distal leaflets adjacent to the inoculated terminal leaflets also were blighted.

V. ABT002 against *P. infestans* on potted potato plants

ABT002 provided good control of *P. infestans* on potato plants (Figure 19). Potted potato plants treated with 50.0 mg/ml of ABT002 resulted in a 100 % control of late blight, statistically comparable with that of the commercial fungicide, chlorothalonil which was applied at the rate of 10.0 mg/ml (Figure 18b, Appendix VI). There was no sign of white mycelium or black lesions on the inoculated area of the leaflet surface. Treatment with the lower concentration of ABT002 provided only partial control with up to 58.0 % of the inoculated potato leaf infected with *P. infestans*, although this infection was restricted to the inoculated leaflets. From visual observation, the blighted area of the infected leaflet in this treatment was smaller than that in the controls.

Figure 19: Control of late blight (*Phytophthora infestans*) infection on 4- to 5-week-old, potted potato plants, 8 d after treatment with (a) 10.0 mg/ml (1.0 %) and (b) 50.0 mg/ml (5.0 %) ABT002, compared with that of distilled water (untreated), carrier and 10.0 mg/ml (1.0 %) chlorothalonil.


a
UNTREATED
WHOLE EXTRACT
ABT002 1.0 % (w/v)
COMMERCIAL FUNGICIDE
CHLOROTHALONIL
1.0 % (w/v)
b
UNTREATED
CARRIER
FOR ABT002
whole fraction
ABT002 5.0 % (w/v)

4.0. DISCUSSION

These experiments have shown that the metabolic products of *X. bovienii* have strong, selective antimycotic activity and, for the first time, they have been shown to control *P. infestans* infection of potted plants. These substances have the potential to be developed as agricultural fungicides. The organic (ABT001) and 'whole' (ABT002) fractions provided excellent control of *P. infestans* but not of *P. ultimum* and *R. solani*. However, both fractions caused phytotoxic responses at high concentrations. The considerations and qualifications bearing on these statements will now be discussed.

Xenorhabdus bovienii was selected based on reports that the phase one of this bacterium is more stable than *X. nematophilus* (Chen, 1992) and that it produces metabolic products, including the indole and dithiolopyrrolone derivatives, that possess antimycotic activity (Li *et al.*, 1995). Aside from *X. bovienii*, *X. nematophilus* and an undescribed *Xenorhabdus* sp. strain Q1, there is no information on the nature of the antibiotic compounds from other species of *Xenorhabdus*. The phase one variant of *X. bovienii* A2 was maintained by regular subculturing in nutrient agar, and this agrees with Ehlers *et al.* (1990) who reported that the shift from phase one to phase two is probably the result of nutrient deficiency.

Production of an inoculum from *X. bovienii* on solid agar media was not feasible due to the varying growth rates of the bacterium and the differing size of the inoculum required. Therefore, a liquid medium was used. The size of the inoculum from the NBTA plate was crucial as it determined the duration of the bacterial culture that was required to reach mid-exponential growth phase. Significantly decreased or completely inhibited bacterial growth can lead to phase

variation (Akhurst and Dunphy, 1993) and, consequently, the inoculum culture in my experiments was not allowed to exceed mid-exponential growth phase.

All fermentation cultures were consistently harvested at 96 h so as to standardize as much as possible the level of antibiotic activity of the culture supernatant. Moreover, the antibacterial and antimycotic activity of both the culture supernatant and the organic fraction were among the highest at 96 h. Interestingly, Li and Chen using high performance liquid chromatography found that the total amount of indoles produced by *X. bovienii* A2 was highest at 96 h (personal communication). Similar results were reported by Sundar and Chang (1993) for *X. nematophilus*, where antibiotic accumulation in the culture medium reached a peak at the late stationary phase when monitored over 96 h.

The extraction process for the organic fraction, using ethyl acetate is a standard procedure. A new procedure was developed to extract the crude antibiotic substance from the culture supernatant or the aqueous fraction (Li, personal communication). Amberlite XAD-2, a hydrophobic polyaromatic resin that 'absorbs' metabolites through hydrophobic interaction, was used in the column and the 1:1:1 mixture of distilled water:butanol:acetic acid was used to 'release' metabolites from the resins. The 1:1:1 eluate did not contain all the active metabolites present in the culture supernatant or aqueous fraction as the water eluates were found to have antimicrobial activity. The dried 1:1:1 eluate from the culture supernatant was labeled as 'whole' extract (ABT002). Although this fraction does not consist of all the active metabolites present in the culture supernatant, it is believed to contain most of the antibiotic substances present in both organic and aqueous fractions. No antibiotic compounds have been reported from the aqueous fraction although it has both antibacterial and antimycotic activity. This is not uncommon because most antibiotics produced by bacteria or fungi are isolated from the organic fraction due to the fact that the extraction and

isolation processes are less complex and most cost efficient. Some examples of antimycotic metabolites isolated from organic fractions are the pyrrolnitrin and pyoluteorin from *Pseudomonas fluorescens* (Howell and Stipanovic, 1979, 1980), faeriefungin from *Streptomyces griseus* var. autotrophicus (Smith *et al.*, 1990) and chaetomin from *Chaetomium globosum* (Di Pietro *et al.*, 1992).

There was some concern that the level of production of antibiotics and their bioactivity might change over time with repeated subculturing of the bacterium. The data on consistency in the level of production and of the activity of the antibiotics of the phase one variant of *X. bovienii* A2 throughout this project provided a reasonable confidence level. However, due to the limited nature of the data on the antimycotic activity of these cultures it is uncertain if there was change in the level of production or activity of the antimycotic substances over time even though the limited data available did not suggest otherwise. *Bacillus subtilis* was used as a bioassay from the beginning of this project as it is a fast and efficient bioassay of the level of antibacterial activity in the cultures. Subsequently, *B. cinerea* was developed as the bioassay during that part of the project where most of the experiments were conducted.

Use of the lyophilized supernatant for fungal control was shown to be impractical as it was phytotoxic to plants. Since plants treated with TSB expressed phytotoxic symptoms similar to those in plants treated with the lyophilized supernatant, it is believed that the high concentration of the available nutrients in TSB (see Appendix VII) and the supernatant might be responsible for the phytotoxic damage. Moreover, there is no report on the use of any lyophilized culture supernatant for the control of fungal diseases in plants.

Pythium ultimum and *R. solani* were selected as test fungi because they are economically important pathogens in B.C., and the mycelial growth of both fungi was completely inhibited in preliminary Petri plate experiments by antimycotic

substances produced by the phase one variant of *X. bovienii* (Chen *et al.*, 1994). *Phytophthora infestans* (A1 mating type) was chosen because my preliminary, Petri plate studies showed that mycelial growth of *P. infestans* was strongly inhibited in the presence of 96 h culture of *X. bovienii*.

Although *P. infestans* and *P. ultimum* are from the same order, Peronosporales, the organic soluble, antimycotic substance (ABT001) was effective against *Phytophthora* and not against *Pythium*. Similarly, this selectivity is observed in the commercially formulated fungicide, chlorothalonil, which is fungicidal against *P. infestans* but not against *P. ultimum*. However, high concentrations of the organic soluble, antimycotic substance were fungistatic against *P. ultimum* and *R. solani*. Interestingly, the antimycotic substance from the same strain of *X. bovienii* was reported to completely inhibit mycelial growth of *P. ultimum* and *R. solani* in Petri plate culture using live bacteria (Chen *et al.*, 1994). *Phytophthora infestans* was not tested by these workers, but the difference of my results from theirs for *P. ultimum* could be explained either by removal or inactivation of the active antimycotic compound(s) during the extraction process. The antimycotic compound(s) responsible for inhibition of *P. ultimum* might be present in the aqueous fraction and not in the organic fraction. Although the growth of the mycelium was negatively influenced by concentrations of each of the three fractions, there was a very distinct difference in appearance of their respective mycelial mats. The mycelial mats treated with the water soluble, antimycotic substance (ABT003) were sparse whereas those treated with the organic soluble, antimycotic substance (ABT001) were dense and compact. Chen *et al.* (1995) reported that *X. bovienii* A2 produces enzymes with chitinolytic activity, and this might explain the ability of this bacterium to completely inhibit mycelial growth of *R. solani* in a dual culture test. Unlike *P. infestans* and *P. ultimum*, chitin is present in the cell wall of *R. solani* (Webster, 1980). The

inability of the extracts to inhibit mycelial growth of *R. solani* when assayed on Petri plates in my experiments may be associated with the loss or damage of the enzymes during the extraction process.

Two isolates of *P. infestans* of the A1 mating type, one metalaxyl sensitive and one metalaxyl insensitive, were initially tested on Petri plates with the culture supernatant. Both strains were extremely sensitive to metabolic products from *X. bovienii*. Since metalaxyl has been one of the standard control fungicides for *P. infestans*, the metalaxyl sensitive isolate was selected in these tests. However, mycelial growth of *P. infestans* was totally inhibited by organic soluble, antimycotic substance and by chlorothalonil, but not by metalaxyl. This was surprising, knowing that this isolate of *P. infestans* was metalaxyl sensitive. When the parent culture was tested with metalaxyl similar results were obtained, which led to the assumption that there was an error in labeling during the isolation process. Metalaxyl is a systemic, phenylamide fungicide that suppresses lesion expansion, sporulation and sporangial viability of *P. infestans* (Bruck *et al*, 1980). The development of resistance to metalaxyl has been documented in Europe (Carter *et al.*, 1982; Davidse *et al.*, 1981), the Middle East (Cohen and Reuveni, 1983), Mexico (Matuszak *et al.*, 1994) and Canada (Chycoski and Punja, 1995).

Four indole derivatives and seven xenorhabdins have been isolated from the organic fraction of the culture supernatant of *X. bovienii* (McInerney et al., 1991a, Li *et al.*, 1995). The four indole derivatives are 3-(2'-acetoxy-3'-keto-4'-methylhexyl)-indole, 3-(2'-acetoxy-3'-keto-4'-methylpentyl)-indole, 3-(2'-hydroxy-3'-keto-4'-methylhexyl)-indole, and 3-(2'-hydroxy-3'-keto-4'-methylpentyl)-indole (Li *et al.*, 1995) (Appendix VIII). Although the mode of action of these metabolites on fungi is unknown, their mechanism of action on bacteria has been studied. Sundar and Chang (1993) reported that the indole antibiotics suppress the growth of a wide range of Gram-positive and Gram-negative bacteria by

increasing the intracellular level of the regulatory nucleotide, guanosine-3', 5'-bis-pyrophosphate (ppGpp) which in turn leads to severe inhibition of net RNA synthesis accompanied by a less severe effect on protein synthesis. Although similar work has not been conducted on fungi, a similar mode of action might occur in this group of microorganisms.

Xenorhabdins contain the pyrrothine ring, and similar compounds including thiolutin and aureothricin (Celmer and Solomons, 1955), holomycin (Ettinger *et al.*, 1959) and isobutyropyrrothine (Bhate *et al.*, 1960), which have the same ring system have been isolated from *Streptomyces* spp. (Appendix IX). Although the xenorhabdin bioactivity spectrum has not been tested intensively, its related pyrrothines are known to possess high activity against a variety of fungi, amoeboid parasites, and Gram-negative and Gram-positive bacteria (Celmer and Solomons, 1955; Bhate *et al.*, 1960; Jimenez *et al.*, 1973). The fungicidal activity observed on *P. infestans* could be due partly to the presence of xenorhabdins. Although the xenorhabdin mode of action is unknown, a related compound, thiolutin, is a potent RNA polymerase inhibitor and known to block the transcription process in yeast cells (Tipper, 1973; Jimenez *et al.*, 1973). Therefore, the mode of action of the antimycotic compounds of *X. bovienii* could be similar to that of these two groups of antibiotics, and the apparent difference in susceptibility of the three test fungi observed in this experiment may be associated with the different rates of penetration/absorption of these compounds through the fungal cell wall.

Based on the promising Petri plate results, the antimycotic substances (ABT001 and ABT002) were tested on potted potato plants. However, in the initial attempts to do so in this experiment there were difficulties in achieving close to a 100 % late blight infection of the potato plants. The method described by Bruck *et al.* (1981) was tried where potted plants were sprayed to run-off with a suspension of sporangia at the rate of 10,000 /ml before being placed in a 100 %

relative humidity growth chamber at 19 °C. However, only sporadic blight lesions were observed when the plants were assessed after 7 d of incubation. Initially, the potato cultivar being used was suspect because there are differences in susceptibility of potato cultivars to late blight. When three potato cultivars, Norchip, Norgold and Yukon Gold, were tested similar problems of poor late blight infections were observed with all three cultivars. The inability of the late blight infection to develop was thought to be caused by using an incorrect temperature because temperature is known to play an important role in development of this disease on potato leaves. Stevenson (1993) stated that sporangia germinate by two different methods, depending on the temperature; between 21 and 25 °C, they germinate directly, forming a germ tube that penetrates the host tissues. Below 18-19 °C, the contents of each sporangium divide into six to eight zoospores for which the optimum temperature for germination is 12-15 °C. He also emphasized that once host tissues have become infected, the optimum temperature for fungal growth within the host is 18-22 °C. Therefore, by having the temperature at 19 °C throughout the whole experiment, this environment probably induced the sporangia to produce zoospores which then did not germinate.

Another suspected experimental variable that might have inhibited late blight infection in the growth chamber was the air circulation. Zoospores are sensitive to drying and they need a film of water on the leaf surface for successful infection. Taking these factors into consideration, a couple of trials were conducted where inoculated plants were covered with transparent polyethylene bags and maintained at 16-20 °C. Inoculation was performed by placing 10 µl of sporangial suspension (5,000 sporangia/ml) on the abaxial surface of the terminal leaflets, as described by Bruck *et al.* (1981). This increased the percentage of successful infection on inoculated leaflets, but did not achieve 100 % infection.

Additional trials using different temperature regimes and inocula, suspensions of zoospores and mycelial plugs, were conducted without success. Only 40-60 % of the inoculated leaflets developed blighted lesions, and there was a wide variation in the lesion size on the infected leaflets. Finally, after further trials, successful infections were consistently obtained on potato leaflets that had a constant water film on the surface. Therefore, trials were conducted where the foliage of the potato plants was sprayed with a fine mist of water before inoculation and again after 48 h of incubation, as described in section 3.4.3-II. This wetting of the leaflets resulted in a consistent 100 % infection on the inoculated leaflets, and the production of lesions of similar size. The infected leaflets had the characteristic enlarged brown or purplish black lesion with cottonlike white mycelium on the under surface of the leaflet. The range of temperature, 16-20 °C favoured the development of zoospores from sporangia, zoospore germination and fungal growth, which explains the necessity of having a consistent water film on the foliage surface over a sustained period.

The results obtained from the potted plant tests confirmed those of the preliminary Petri plate tests. The organic soluble antimycotic substance achieved the same level of control of late blight development as did the chlorothalonil treatment. Chlorothalonil is a broad spectrum, protectant fungicide that inhibits zoospore and sporangium germination (Bruck *et al.*, 1981). With no infection lesions present, it is possible that the antimycotic substance produced by *X. bovienii* inhibits directly zoospore or sporangium germination. Interestingly, lower concentrations of this substance reduces the size and growth of the blight lesions. The antimycotic substance in the 'whole' fraction also controlled late blight development, but only at higher concentrations. The water soluble, antimycotic substance was fungicidal against *P. infestans* at high concentrations, as was observed in the Petri plate test and together with the organic soluble,

antimycotic substance present in the 'whole' fraction these substances could be responsible for its overall antimycotic activity. From these observations, it could be concluded that the antimycotic compounds which have fungicidal activity against *P. infestans* could be present also in the aqueous fraction. The inability of the antimycotic substance from the organic fraction to control *P. ultimum* and *R. solani* in Petri plate tests was substantiated when this substance failed as a protectant when tested on bush bean seed and potted grass, respectively.

Although the antimycotic substance in both the organic and 'whole' fractions provided excellent control against *P. infestans* on potato plants, these fractions were phytotoxic at high concentrations. However, the phytotoxic responses on potato plants treated with up to 10 mg/ml ABT001 or 50 mg/ml ABT002 were not permanent as treated plants grew away from the damage. The precise phytotoxic property of these substances is unknown but, in part, it could be due to the indole compounds. The antibiotic compounds of *X. bovienii* have the same indole ring as the indole-3-acetic acid (IAA) (Appendix X), a natural plant growth hormone, and the antibiotic indole compounds and the IAA are derived from the same precursor, tryptophan (Sembdner *et al.*, 1980; Sundar and Chang, 1993). High concentrations of IAA stimulate the biogenesis of ethylene in plant tissues (Burg and Burg, 1966; Yang and Hoffman, 1984). Ethylene is known to inhibit cell division, and the production and action of this plant hormone in some but not all situations are linked to the continuous presence of IAA. The classic example is the inhibition of elongation by high concentrations of IAA which results in the production of ethylene (Matthysse and Scott, 1984). The experiment on bush bean seed sheds some light on the phytotoxic properties of the antimycotic substance. The stunted physical appearance and the reduced length of the seedling hypocotyl may be explained by the high concentration of indole compounds in the antimycotic substance. A similar explanation could be applied

to the delay in germination in the same experiment, because ethylene or high concentrations of IAA is known to inhibit or delay the opening of the hypocotyl hook (Matthysse and Scott, 1984). The coiling appearance of the treated bush bean hypocotyl resembled the wrong-way, gravitropic curvature described by Harrison and Pickard (1989) in an experiment where seedlings were treated with elevated levels of IAA. Interestingly, the epinastic symptoms observed on the antimycotic treated potato plants were similar to those described for tomato plants treated with high levels of IAA and ethylene (Leather *et al.*, 1972; Kazemi and Kefford, 1974; Kang, 1979), where the IAA action is dependent upon induction of ethylene synthesis (Amrhein and Schneebeck, 1980). Although there is no conclusive data to support the hormonal property of these indole compounds or their bioactivity in plant tissues, the similarity of the observations on bush bean seedlings and potato plants with those reported for exogenous application of IAA and ethylene are sufficient to render the suggestion that the indole compounds in the antimycotic substance cause the phytotoxic symptoms. As well, IAA has been known to have inhibitory effect on fungi (Matthysse and Scott, 1984).

The excellent antimycotic activity of the metabolites of *X. bovienii* has been demonstrated here against *P. infestans*. Although these substances possess phytotoxic properties at high concentration, it should not be an obstacle for further identifying the responsible antimycotic compound(s) and doing field tests. The indole and the xenorhabdin compounds are probably not the only compounds responsible for the antimycotic activity in these various metabolic fractions. However, it is unlikely that these crude antimycotic substances could be used for fungal disease control on living potato plants because of their uncertain phytotoxic properties. Moreover, this is made progressively more difficult by the increasingly stringent registration requirements for the application of microbial products. These require precise information on the physical, biological and

chemical properties of the metabolites as well as on the mode of action on the target species. However, as has happened to many promising antimycotic metabolites (Rodgers, 1989; Beautement *et al.*, 1991; Pillmoor *et al.*, 1993; Lange *et al.*, 1993), these antimycotic compounds produced by *X. bovienii* may be used as lead molecules for the synthesis of new fungicides. As well, if the hormonal activity of the indole compounds is proven, this property could be an added value to their now proven antimycotic activity. A new group of fungicides that included a growth factor may be developed if such lead molecules could be manipulated appropriately.

However, by no means all the available knowledge and technology on this bacterium should be restricted to the agrochemical companies. This research answers some intriguing questions on the biology of *X. bovienii* and its active metabolites as well as raising additional questions. More information is needed on the biological activity of these bacterial metabolites and especially on the antimycotic spectrum and precise mode of action of the isolated compounds. Since the antimycotic substance has proven effective against *P. infestans*, it would be useful to examine its activity against other *Phytophthora* species. As well, a standardized procedure should be developed to culture *X. bovienii* in large quantities as it is time consuming and impractical to produce large quantities of biologically active metabolites in a 2,000 ml flask, even for testing. *Xenorhabdus bovienii* is, indeed, an unique bacterium and it may well provide some answers to our increasing problems in plant disease management in the near future.

5.0. REFERENCES

Adams, P. B. (1990). The potential of mycoparasites for biological control of plant diseases. *Annual Review of Phytopathology* 28, 59-72.

Adams, P. B. and Ayers, W. A. (1982). Biological control of *Sclerotinia* lettuce drop in the field by *Sporidesmium sclerotivorum*. *Phytopathology* 72, 485-488.

Aguillera, M. M., Hodge, N. C., Stall, R. E. and Smart, G. C. Jr. (1993). Bacterial symbionts of *Steinernema scapterisci*. *Journal of Invertebrate Pathology* 62, 68-72.

Akhurst, R. J. (1980). Morphological and physiological dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes, *Neoaplectana* and *Heterorhabditis*. *Journal of General Microbiology* 121, 303-309.

Akhurst, R. J. (1982). Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. *Journal of General Microbiology* 128, 3061-3065.

Akhurst, R. J. (1986). *Xenorhabdus nematophilus* subsp. *poinarii*: its interaction with insect pathogenic nematodes. *Systematic and Applied Microbiology* 8, 142-147.

Akhurst, R. J. and Boemare, N. E. (1988). A numerical taxonomic study of the genus *Xenorhabdus* and the proposed elevation of the subspecies of *X. nematophilus* to species. *Journal of General Microbiology* 134, 1835-1845.

Akhurst, R. J. and Boemare, N. E. (1990). Biology and taxonomy of *Xenorhabdus*. In *Entomopathogenic Nematodes in Biological Control*. Gaugler, R. and Kaya, H. K. (Eds.). pp. 75-90. CRC Press, St. Paul, Minnesota.

Akhurst, R. J. and Dunphy, G. B. (1993). Tripartite interactions between symbiotically associated entomopathogenic bacteria, nematodes, and their insect hosts. In *Parasites and Pathogens of Insects, Vol. 2: Pathogens*. Beckage, N. E., Thompson, S. N. and Federici, B. A. (Eds.). pp. 1-23. Academic Press Inc., San Diego.

Albert, G., Curtze, J. and Drandarevski, C. A. (1988). Dimethomorph (CME151) - a novel curative fungicide. In *Brighton Crop Protection Conference;*

Pests and Disease-1988, Vol. 1. pp. 17-24. British Crop Protection Council, Thornton Heath, UK.

Ammermann, E., Locher, F., Lorenz, G., Janssen, B., Karbach, S. and Meyer, N. (1990). BAS 480.F - a new broad spectrum fungicide. In *Brighton Crop Protection Conference; Pests and Disease-1990, Vol. 2.* pp. 407-414. British Crop Protection Council, Surrey, UK.

Ammermann, E., Lorenz, G., Schelberger, K., Wenderoth, B., Sauter, H. and Rentzea, C. (1992). BAS 490F - a broad spectrum fungicide with a new mode of action. In *Brighton Crop Protection Conference; Pests and Disease-1992, Vol. 1.* pp. 403-410. British Crop Protection Council, Farnham, UK.

Amrhein, N. and Schneebeck, D. (1980). Prevention of auxin-induced epinasty by α-aminooxyacetic acid. *Physiology of Plants* 49, 62-64.

Anke, T., Besl, H., Mocek, U. and Steglish, W. (1983). Antibiotics from Basidiomycetes: 18. Strobilurin C and oudemansin B, 2 new antifungal metabolites from *Xerula* spp. (Agaricales). *Journal of Antibiotics* 36, 661-666.

Anke, T., Hecht, H. J., Schramn, G and Steglich, W. (1979). Antibiotic from Basidiomycetes: 9. Oudemansin, an antifungal antibiotic from *Oudemansiella mucida* (Agaricales). *Journal of Antibiotics* 32, 1112-1117.

Anke, T., Werle, A., Bross, M. and Steglich, W. (1990). Antibiotic from Basidiomycetes: XXXIII. Oudemansin X, a new antifungal E-β-methoxyacrylate from *Oudemansiella radicata* (Agaricales). *Journal of Antibiotics* 43, 1010-1011.

Anonymous (1960). *Index of Plant Diseases in the United States. USDA Handbook No. 165.* Crops Research Division, ARS, USDA, Washington DC. 531 pp.

Anonymous (1985). *The World Agrochemical Market.* Agrow World Agrochemical News. George Street Publications Ltd., Richmond, Surrey.

Anonymous (1987a). *Agrochemical Service.* Wood-Mackenzie, Edinburgh, UK.

Anonymous (1987b). *Regulating Pesticides in Food.* Washington, DC; National Academy Press. 288 pp.

Audus, L. J. (1972). *Plant Growth Substances. Vol. 1: Chemistry and Physiology.* Leonard Hill Books, London. 533 pp.

Ayers, W. A. and Lumsden, R. D. (1975). Factors affecting production and germination of oospores of three *Pythium* species. *Phytopathology* 65, 1094-1100.

Beautement, K., Clough, J. M., de Fraine, P. J. and Godfrey, C. R. A. (1991). Fungicidal β-methoxyacrylates: from natural products to novel synthetic agricultural fungicides. *Pesticide Science* 31, 499-519.

Bhate, D. S., Hulyalkar, R. K. and Menon, S. K. (1960). Isolation of *iso*-butyropyrrothine along with thiolution and aureothricin from a *Streptomyces* sp.. *Experientia* 16, 504.

Blaxter, K. (1986). Food and people. *Human Nutrition : Clinical Nutrition* 40C, 95-118.

Boemare, N. E. and Akhurst, R. J. (1988). Biochemical and physiological characterization of colony from variants in *Xenorhabdus* spp. (Enterobacteriaceae). *Journal of General Microbiology* 134, 751-761.

Boemare, N. E., Akhurst, R. J. and Mourant, R. G. (1993). DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov.. *International Journal of Systemic Bacteriology* 43, 249-255.

Bruck, R. I., Fry, W. E. and Apple, A. E. (1980). Effect of metalaxyl, an acylalanine fungicide, on developmental stages of *Phytophthora infestans*. *Phytopathology* 70, 597-601.

Bruck, R. I., Fry, W. E., Apple, A. E and Mundt, C. C. (1981). Effect of protectant fungicides on developmental stages of *Phytophthora infestans* in potato foliage. *Phytopathology* 71, 164-166.

Burg, S. P. and Burg, E. A. (1966). The interaction between auxin and ethylene and its role in plant growth. *Proceedings of the National Academy of Science, U.S.A.* 55, 262-269.

Cabanillas, H. E., Poinar, G. O. and Raulston, J. R. (1994). *Steinernema riobravis* n. sp. (Rhabditida:Steinernematidae) from Texas. *Fundamental and Applied Nematology* 17, 123-131.

Carter, G. A., Smith, R. M., and Brent, K. J. (1982). Sensitivity to metalaxyl of *Phytophthora infestans* in potato crops in south-west England in 1980 and 1981. *Annals of Applied Biology* 100, 433-441.

Celmer, W. D. and Solomons, I. A. (1955). The structures of thiolutin and aureothricin, antibiotics containing a unique pyrrolinonodithiole nucleus. *Journal of American Chemical Society* 77, 2861-2865.

Chen, G. (1992). Effects of antibiotics produced by *Xenorhabdus* spp. on soil bacteria. M.S. thesis. Simon Fraser University, Burnaby, British Columbia, Canada.

Chen, G., Dunphy, G. B. and Webster, J. M. (1994). Antifungal activity of two *Xenorhabdus* species and *Photorhabdus luminescens* species and *Heterorhabditis megidis*. *Biological Control* 4, 157-162.

Chen, G., Zhang, Y., Dunphy, G. B., Punja, Z. K. and Webster, J. M. (1995). Chitinolytic enzyme activity of *Xenorhabdus* and *Photorhabdus* species. *Microbiology* (submitted).

Chycoski, C. I. and Punja, Z. K. (1995). Characterization of populations of *Phytophthora infestans* from potato in Canada during 1993 and 1994. *Plant Disease* (submitted).

Cohen, Y., and Reuveni, M. (1983). Occurrence of metalaxyl resistant isolates of *Phytophthora infestans* in potato fields in Israel. *Phytopathology* 73, 925-927.

Currier, T. C., Skwara, J. E., and McIntyre, J. L. (1988). The development of a *Pseudomonas fluorescens* product (Dagger®) for the control of *Pythium* and *Rhizoctonia* on cotton. In *1988 Proceeding of the Beltwide Cotton Producer and Research Conference*. pp. 18-19. National Cotton Council and the Cotton Foundation, Memphis, Tennessee.

Couche, G. A., Lehrbach, P. R., Forage, R. G., Cooney, G. C., Smith, D. R. and Gregson, R. P. (1987). Occurrence of intracellular inclusions and plasmids in *Xenorhabdus* spp. *Journal of General Microbiology* 133, 967-973.

Davidse, L. C., Looijen, D., Turkensteen, L. J., and Van der Wal, D. (1981). Occurrence of metalaxyl-resistant strains of *Phytophthora infestans* in Dutch potato fields. *Netherlands Journal of Plant Pathology* 87, 65-68.

Deb, P. R. and Dutta, B. K. (1984). Activity of thiolutin against certain soil-borne plant pathogens. *Current Science* 53, 659-662.

Di Pietro, A., Gut-Rella, M., Pachlatko, J. P. and Schwinn, F. J. (1992). Role of antibiotics produced by *Chaetomium globosum* in biocontrol of *Pythium ultimum*, a causal agent of damping-off. *Phytopathology* 82, 131-135.

Driant, D., Hede-Hauy, L., Perrot, A., Quinn, J. A. and Shaber, S. A. (1988). RH 7592 - a new triazole fungicide with high specific activity on cereals and other crops. In *Brighton Crop Protection Conference; Pests and Disease-1988, Vol. 1.* pp. 33-40. British Crop Protection Council, Thornton Heath, UK.

Dutky, S. R. (1974). Nematode parasites. In *Proceedings of the Summer Institute on Biological Control of Plant Insects and Diseases*. Maxwell, F. G. and Harris F. A. (Eds.). pp. 576-590. University Press of Mississippi, Jackson, Mississippi.

Dutky, S. R., Thompson, J. V. and Cantwell, G. E. (1964). A technique for the mass propagation of the DD-136 nematode. *Journal of Insect Pathology* 6, 417-422.

Eckert, J. W. (1988). Historical development of fungicide resistance in plant pathogens. In *Fungicide Resistance in North America*. Delp, C. J. (Ed.). pp. 1-3. APS Press, St. Paul, Minnesota.

Ehlers, R. U., Stoessel, S. and Wyss, U. (1990). The influence of phase variants of *Xenorhabdus* spp. and *Escherichia coli* (Enterobacteriaceae) on the propagation of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*. *Revue de Nematologie* 13, 417-424.

Ettinger, L., Guamann, E., Hutter, R., Keller-Schierlein, W., Kradolfer, F., Neipp, L., Prelog, V. and Zahner, H. (1959). Stoffwechselprodukte von actinomycetes 17. Holomycin [Actinomycetes metabolites 17. Holomycin]. *Helvetica Chimica Acta* 42, 563-569.

Finney, J. R. (1988). World crop protection: Demisting the crystal ball. In *Brighton Crop Protection Conference; Pests and Disease-1988, Vol. 1.* pp. 3-14. British Crop Protection Council, Thornton Heath, UK.

Gehmann, K., Nyfeler, R., Leadbeater, A. J., Nevill, D. and Sozzi, D. (1990). CGA 173506: A new phenylpyrrole fungicide for broad-spectrum disease control, In *Brighton Crop Protection Conference; Pests and Disease-1990, Vol. 2.* pp. 399-406. British Crop Protection Council, Surrey, UK.

Godwin, J. R., Anthony, V. M., Clough, J. M. and Godfrey, C. R. A. (1992). ICIA5504 - A novel broad spectrum systemic β-methoxyacrylate fungicide In *Brighton Crop Protection Conference; Pests and Disease-1992, Vol. 1.* pp. 435-442. British Crop Protection Council, Farnham, UK.

Griffiths, W. (1988). Crop production realism: the role of agrochemicals. In *Brighton Crop Protection Conference; Pests and Disease-1988, Vol. 1.* pp. 111-120. British Crop Protection Council, Thornton Heath, UK.

Grimont, P. A. D., Steigerwalt, A. G., Boemare, N. E., Hickman-Brenner, F. W., Deval, C., Grimont, F. and Brenner, D. J. (1983). Deoxyribonucleic acid relatedness and phenotypic study of the genus *Xenorhabdus*. *International Journal of Systematic Bacteriology* 34, 378-388.

Harrison, M. A. and Pickard, B. G. (1989). Auxin asymmetry during gravitropism by tomato hypocotyls. *Plant Physiology* 89, 652-657.

Heaney, S. P., Hofman, T. W. and Grosscurt, A. C. (1988). ICI A0001 - a novel benzamide fungicide. In *Brighton Crop Protection Conference; Pests and Disease-1988, Vol. 2.* pp. 551-558. British Crop Protection Council, Thornton Heath, UK.

Hewitt, W. and Vincent, S. (1989). *Theory and Application of Microbiological Assay.* Academic Press, San Diego. 323 pp.

Hofman, T. W., Kuipers, J. and Zech, B. (1990). Thicyofen: a new broad-spectrum fungicide for seed and soil treatment. In *Brighton Crop Protection Conference; Pests and Disease-1990, Vol. 2.* pp. 431-438. British Crop Protection Council, Surrey, UK.

Howell, C. R. and Stipanovic, R. D. (1979). Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. *Phytopathology* 69, 480-482.

Howell, C. R. and Stipanovic, R. D. (1980). Suppression of *Pythium*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. *Phytopathology* 70, 712-715.

James, W. C. (1981). Estimated losses of crops from plant pathogens. In *Handbook of Pest Management in Agriculture, Vol. 1.* Pimentel, D. (Ed.). pp. 79-94. CRC Press, Boca Raton, Florida.

Jeffers, S. N. and Martin, S. B. (1986). Comparison of two media selective for *Phytopthora* and *Pythium* species. *Plant Disease* 70, 1038-1043.

Jewell, L. D. (1987). *Agricultural Statistics, 1987.* Washington, DC; US Government Printing Office. 541 pp.

Jimenez, A., Tipper, D. J. and Davies, J. (1973). Mode of action of thiolutin, an inhibitor of macromolecular synthesis in *Saccharomyces cerevisiae*. *Antimicrobial Agents and Chemotherapy* 3, 729-738.

Kang, B. G. (1979). Epinasty. In *Encyclopedia of Plant Physiology, Vol. 7.* Haupt, W. and Feinlab, M. E. (Eds.). pp. 647-667. Springer-Verlag, Berlin.

Kazemi, S. and Kefford, N. P. (1974). Apical correlative effects in leaf epinasty of tomato. *Plant Physiology* 54, 512-519.

Lange, L., Breinholt, J., Rasmussen, F.W., and Nielsen, R.I. (1993). Microbial fungicides - the natural choice. *Pesticide Science* 39, 155-160.

Leather, G. R., Forrence, L. E. and Abeles, F. B. (1972). Increased ethylene production during clinostat experiments may cause leaf epinasty. *Plant Physiology* 49, 183-186.

Lethbridge, G. (1989). An industrial view of microbial inoculants for crop plants. In *Microbial Inoculation of Crop Plants*. Campbell, R. and Macdonald, R. M. (Eds.). pp. 11-28. IRL Press, Oxford.

Lewis, J. A. and Papavizas, G. C. (1991). Biocontrol of plant diseases: the approach for tomorrow. *Crop Protection* 10, 95-105.

Li, J., Chen, G. and Webster, J. M. (1995). Antimicrobial metabolites from a bacterial symbiont. *Journal of Natural Products* (accepted).

Margot, P., Eckhardt, W. and Dahmen, H. (1988). CGA 80000 - a new phenylamide fungicide against soil-borne Peronosporales. In *Brighton Crop Protection Conference; Pests and Disease-1988, Vol. 2.* pp. 527-534. British Crop Protection Council, Thornton Heath, UK.

Matthysse, A. G. and Scott, T. K. (1984). Function of hormones at the whole plant level of organization. In *Hormonal Regulation of Development II; The Function of Hormones from the level of the cell to the whole plant. Encyclopedia of Plant Physiology, New Series Vol. 10.* Scott, T. K. (Ed.). pp. 219-243. Springer-Verlag, Berlin.

Matuszak, J. M., Fernandez-Elquezabal, J., Gu, W. K., Villarreal-Gonzalez, M. and Fry, W. E. (1994). Sensitivity of *Phytophthora infestans* populations to metalaxyl in Mexico: Distribution and dynamics. *Plant Disease* 78, 911-916.

Maxwell, P. W., Chen, G., Webster, J. M. and Dunphy, G. B. (1994). Stability and activities of antibiotics produced during infection of the insect *Galleria mellonella* by two isolates of *Xenorhabdus nematophilus*. *Applied and Environmental Microbiology* 60, 715-721.

McInerney, B. V., Taylor, W. C., Lacey, M. J., Akhurst, R. J. and Gregson R. P. (1991a). Biologically active metabolites from *Xenorhabdus* spp. Part II:

Benzopyran-1-one derivatives with gastroprotective activity. *Journal of Natural Products* 54, 785-795

McInerney, B. V., Gregson, R. P., Lacey, M. J., Akhurst, R. J., Lyons, G. R., Rhodes, S. H., Smith, D. R. J., Engelhardt, L. M. and White, A. H. (1991b). Biologically active metabolites from *Xenorhabdus* spp. Part I: Dithiolopyrrolone derivatives with antibiotic activity. *Journal of Natural Products* 54, 774-784.

Mracek, Z., Hernandez, E. A. and Boemare, N. E. (1994). *Steinernema cubana* sp. n. (Nematoda:Rhabditida:Steinernematidae) and the preliminary characterization of its associated bacterium. *Journal of Invertebrate Pathology* 64, 123-129.

Murabayashi, A., Masuko, M., Shirane, N., Hayashi, Y. and Makisumi, Y. (1990). SSF-109, a novel triazole fungicide: Synthesis and biological activity. In *Brighton Crop Protection Conference; Pests and Disease-1990, Vol. 2.* pp. 423-430. British Crop Protection Council, Surrey, UK.

Nevill, D., Nyfeler, R. and Sozzi, D. (1988). CGA 142705 - a novel fungicide for seed treatment. In *Brighton Crop Protection Conference; Pests and Disease-1988, Vol. 1.* pp. 65-72. British Crop Protection Council, Thornton Heath, UK.

Nishimura, Y., Hagiwara, A. and Yamanaka, S. (1994). *Xenorhabdus japonicus* sp. nov. associated with the nematode *Steinernema kushidai*. *World Journal of Microbiology and Biotechnology* 10, 207-210.

Nguyen, K. B. and Smart, G. C. Jr. (1992). *Steinernema neocurtillis* n. sp. (Rhabditida:Steinernematidae) and a key to species of the genus *Steinernema*. *Journal of Nematology* 24, 463-477.

Papavizas, G. C. (1985). *Trichoderma* and *Gliocladium*: Biology, ecology, and potential for biocontrol. *Annual Review of Phytopathology* 23, 23-54.

Papavizas, G. C. and Lewis, J. A. (1988). The use of fungi in integrated control of plant disease. In *Fungi in Biological Control Systems*. Burge, M. N. (Ed.). pp. 235-253. Manchester University Press, Manchester.

Papavizas, G. C. and Lewis, J. A. (1989). Effect of *Gliocladium* and *Trichoderma* on damping-off and blight of snapbean caused by *Sclerotium rolfsii*. *Plant Pathology* 38, 277-286.

Paul, V. J., Frautschy, S., Fenical, W. and Nealson, K. H. (1981). Antibiotics in microbial ecology: isolation and structure assignment of several new

antibacterial compounds from the insect-symbiotic bacteria *Xenorhabdus* spp. *Journal of Chemical Ecology* 7, 589-597.

Pillmoor, J. B., Wright, K. and Terry, A. S. (1993). Natural products as a source of agrochemicals and leads for chemical synthesis *Pesticide Science* 39, 131-140.

Pimentel, D. (1985). Pests and their control. In *CRC Handbook of Natural Pesticides: Methods; Vol. 1, Theory, Practice, and Detection.* Bhushan Mandava, N. (Ed.). pp. 3-19. CRC Press, Boca Raton, Florida.

Poinar, G. O., Hess, R. and Thomas, G. M. (1980). Isolation of defective bacteriophages from *Xenorhabdus* spp. (Enterobacteriaceae). *I.R.C.S. Medical Science* 8, 141.

Poinar, G. O., Mracek, Z. and Doucet, M. M. A. (1988). A re-examination of *Neoaplectana rara* Doucet, 1986 (Steinernematidae:Rhabditida). *Revue de Nematologie* 11, 447-449.

Powell, K. A. and Jutsum, A. R. (1993). Technical and commercial aspects of biocontrol products. *Pesticide Science* 37, 315-321.

Rodgers, P. B. (1989). Potential of biological control organisms as a source of antifungal compounds for agrochemical and pharmaceutical product development. *Pesticide Science* 27, 155-164.

Rowe, G. E. and Margartis, A. (1987). Bioprocess developments in the production of bioinsecticides by *Bacillus thuringiensis*. *Critical Reviews in Biotechnology* 6, 87-127.

Ruess, W., Riebli, P., Herzog, J., Speich, J., and James, J. R. (1988). CGA 169374 - a new systemic fungicide with a novel broad-spectrum activity against disease complexes in a wide range of crops. In *Brighton Crop Protection Conference; Pests and Disease-1988, Vol. 2.* pp. 543-550. British Crop Protection Council, Thornton Heath, UK.

Sanders, P. and Cole, H. Jr. (1986). *In vivo* fungicide screening on greenhouse-grown grasses. In *Methods for Evaluating Pesticides for Control of Plant Pathogens.* Hickey, K. D. (Ed.). pp. 110-111. APS Press, St. Paul, Minnesota.

Sembdner, G., Gross, D., Liebisch, H. W. and Schneider, G. (1980). Biosynthesis and metabolism of plant hormones. In *Hormonal Regulation of Development I; Molecular Aspects of Plant Hormones: Encyclopedia of Plant Physiology, New Series Vol. 9.* MacMillian, J. (Ed.). pp. 281-444. Springer-Verlag, Berlin.

Seneca, H., Kane, J. H. and Rockenbach, J. (1952). Bactericidal, protozoicidal and fungicidal properties of thiolutin. *Antibiotics and Chemotherapy* 2, 357-360.

Smith, J., Putnam, A. and Nair, M. (1990). *In vitro* control of *Fusarium* diseases of *Asparagus officinalis* L. with a *Streptomyces* or its polyene antibiotic, faeriefungin. *Journal of Agricultural and Food Chemistry* 38, 1729-1733.

Stevenson, W. R. (1993). Management of early blight and late blight. In *Potato Health Management*. Rowe, R. C. (Ed.). pp. 141-147. APS Press, St. Paul, Minnesota.

Sundar, L. and Chang, F. N. (1993). Antimicrobial activity and biosynthesis of indole antibiotics produced by *Xenorhabdus nematophilus*. *Journal of General Microbiology* 139, 3139-3148.

Thomas, G. M. and Poinar, G. O. (1979). *Xenorhabdus* gen. nov., a genus of entomopathogenic, nematophilic bacteria of the family Enterobacteriaceae. *International Journal of Systematic Bacteriology* 29, 352-360.

Tipper, D. J. (1973). Inhibition of yeast ribonucleic acid polymerases by thiolutin. *Journal of Bacteriology* 116, 245-246.

Waller, C. D., Eschenbrenner, P. and Godwin, J. R. (1990). Hexaconazole - a new flexible cereal fungicide. In *Brighton Crop Protection Conference; Pests and Disease-1990, Vol. 2.* pp. 447-454. British Crop Protection Council, Surrey, UK.

Webster, J. (1980). *Introduction to Fungi. Second Edition.* Cambridge University Press, Cambridge. 669 pp.

Whipps, J. M., McQuilken, M. P., and Budge, S. P. (1993). Use of fungal antagonists for biocontrol of damping-off and sclerotinia diseases. *Pesticide Science* 37, 309-313.

Woodring, J. L. and Kaya, H. K. (1988). *Steinernematid and Heterorhabditid Nematodes: A Handbook of Biology and Techniques.* Southern Cooperative Series Bulletin 331. Fayetteville, Arkansas. 30 pp.

Yamaguchi, I. (1987). Future prospects for plant protection by pesticides of microbial origin. *Japan Pesticide Information* 50, 17-19.

Yang, S. F. and Hoffman, N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology* 35, 155-189.

APPENDIX I

Antibacterial and antimycotic activity of *Xenorhabdus bovienii* culture supernatant as indicated by the diameter of a clear zone (mm) when bioassayed over a 6 d period with *Bacillus subtillis* and *Botrytis cinerea*, respectively.

	Diameter of clear zone (mm)	
Time (h)	*Bacillus subtilis*	*Botrytis cinerea*
0	0 ± 0 e[#]	0 ± 0 e
24	18.30 ± 0.13 d	10.28 ± 0.20 d
48	20.85 ± 0.13 bc	12.38 ± 0.19 c
72	21.78 ± 0.05 a	12.57 ± 0.14 c
96	21.73 ± 0.18 a	14.38 ± 0.07 a
120	21.10 ± 0.12 b	13.18 ± 0.16 b
144	20.63 ± 0.12 c	12.42 ± 0.23 c

[#]Mean ± standard error of the mean, n = 6. Means with the same(s) letter in the same column are not significantly different ($P < 0.05$).

APPENDIX II

Antibacterial and antimycotic activity of the organic fraction of *Xenorhabdus bovienii* culture supernatant as indicated by the diameter of a clear zone (mm) when bioassayed over a 6 d period with *Bacillus subtillis* and *Botrytis cinerea*, respectively.

	Diameter of clear zone (mm)	
Time (h)	*Bacillus subtillis*	*Botrytis cinerea*
0	0 ± 0 d#	0 ± 0 e
24	13.17 ± 0.38 c	7.10 ± 0.12 d
48	25.62 ± 0.32 b	12.82 ± 0.12 c
72	25.80 ± 0.09 b	14.50 ± 0.15 b
96	26.70 ± 0.35 a	15.73 ± 0.23 a
120	25.33 ± 0.04 b	14.67 ± 0.24 b
144	26.83 ± 0.28 a	15.82 ± 0.39 a

#Mean ± standard error of the mean, n = 6. Means with the same letter in the same column are not significantly different ($P < 0.05$).

APPENDIX III

Effects of three concentrations of ABT001 (mg/g of seed) as seed dressing, on hypocotyl length of bush bean seedling, assessed on day 5 and day 10 after seeding.

	Length of hypocotyl (cm)	
Treatment (per g of seed)	**Day 5**	**Day 10**
Distilled water	6.77 ± 0.19 a[#]	9.54 ± 0.2 a
Carrier only	6.42 ± 0.16 a	8.58 ± 0.17 a
ABT001 0.3 mg	6.65 ± 0.19 a	9.01 ± 0.20 a
ABT001 1.5 mg	5.85 ± 0.19 a	8.47 ± 0.17 a
ABT001 7.5 mg	2.64 ± 0.20 b	3.65 ± 0.22 b

[#]Mean ± standard error of the mean, n = 4 (25 seeds in each replicate). Means with the same letter in the same column are not significantly different ($P < 0.05$) according to Bonferroni's test.

APPENDIX IV

Efficacy of three different concentrations of ABT001 (mg/g of seed) or metalaxyl (mg/g of seed), as seed dressing, on the germination of bush bean seed in soil inoculated with *Pythium ultimum*, assessed on day 5 and 10 after seeding.

	Percentage germination	
Treatment (per g of seed)	**Day 5**	**Day 10**
Distilled water	33.0 ± 13.5 bc[#]	47.0 ± 12.6 b
Carrier only	30.0 ± 5.8 bc	44.0 ± 8.5 b
ABT001 0.3 mg	49.0 ± 4.1 b	62.0 ± 2.6 ab
ABT001 1.5 mg	59.0 ± 6.6 ab	72.0 ± 6.3 ab
ABT001 7.5 mg	7.0 ± 3.4 c	38.0 ± 9.0 b
Metalaxyl 1.5 mg	90.0 ± 4.2 a	96.0 ± 2.8 a

[#]Mean ± standard error of the mean, n = 4 (25 seeds in each replicate). Means with the same letter(s) in the same column are not significantly different ($P < 0.05$) according to Bonferroni's test.

APPENDIX V

Percentage of detached potato leaflet infected with *Phytophthora infestans* in Petri plates 8 d after treatment with one of three concentrations (mg/ml) of ABT001 or ABT002, or with chlorothalonil.

	Percentage of leaflet diseased	
Treatment	**ABT001**	**ABT002**
Distilled water	94.4 ± 3.7 a#	100.0 ± 0 a#
Carrier only	83.3 ± 8.7 a	95.8 ± 4.2 a
ABT001 2.0 mg/ml	33.3 ± 7.1 b	29.2 ± 9.8 b
ABT001 10.0 mg/ml	0 ± 0 c	4.2 ± 4.2 c
ABT001 50.0 mg/ml	0 ± 0 c	0 ± 0 c
Chlorothalonil 10.0 mg/ml	5.6 ± 3.7 c	0 ± 0 c

Mean ± standard error of the mean, n = 10. Means with the same letter in the same column are not significantly different ($P < 0.05$) according to Bonferroni's test.

APPENDIX VI

Percentage of inoculated potato leaflet on 4- to 5-week-old potted potato plant infected with *Phytophthora infestans* 8 d after treatment with one of three concentrations (mg/ml) of ABT001 or ABT002, or with chlorothalonil.

	Percentage of leaflet diseased	
Treatment	**ABT001**	**ABT002**
Distilled water	100 ± 0 a[#]	100 ± 0 a[#]
Carrier only	100 ± 0 a	100 ± 0 a
ABT001 5.0 mg/ml	24.0 ± 5.8 b	nt
ABT001 10.0 mg/ml	4.0 ± 2.7 c	58.0 ± 6.3 b
ABT001 50.0 mg/ml	nt	0 ± 0 c
Chlorothalonil 10.0 mg/ml	0 ± 0 c	0 ± 0 c

[#] Mean ± standard error of the mean, n = 10. Means with the same letter in the same column are not significantly different ($P < 0.05$) according to Bonferroni's test.

nt, not tested

APPENDIX VII

Media Composition and Preparation

All culture nutrients were of cell-culture tested grade and obtained from Difco Laboratories unless otherwise stated. Commercial media were prepared according to the manufacturer's directions and unless otherwise stated, all quantities are the amounts required to mix with one liter of distilled water.

1. Bacto Nutrient Agar (NA)

Bacto - Beef Extract	3 g
Bacto - Peptone	5 g
Bacto - Agar	15 g

2. Bacto Potato Dextrose Agar (PDA)

Potato, Infusion from	200 g
Bacto - Dextrose	20 g
Bacto - Agar	15 g

3. Bacto Tryptic Soy Broth w/o Dextrose (TSB)

Bacto - Tryptone	17 g
Bacto - Soytone	3 g
Sodium chloride	5 g
Dipotassium phosphate	2.5 g

4. Bacto Tryptic Soy Agar w/o Dextrose (TSB)

Bacto - Tryptone	17 g
Bacto - Soytone	3 g
Sodium chloride	5 g
Dipotassium phosphate	2.5 g
Bacto - Agar	15 g

5. NBTA (Woodring and Kaya, 1988)

Bacto Nutrient Agar (NA)	23 g
Bromothymol Blue (BTB)	0.025 g
Triphenyltetrazolium chloride (TTC)	0.04 g

Nutrient agar (NA) and BTB were mixed together and autoclave at 121 °C for 15 minutes. Triphenyltetrazolium chloride was dissolved in a few milliliters distilled water and filter-sterilized through a Millipore filter (0.2 micron). The TTC solution was added into the cooled medium (48 °C), and mixed thoroughly.

6. P_5ARPH (Jeffers and Martin, 1986)

Bacto Corn Meal Agar (CMA)	17 g
Pimaricin	0.005 g
Sodium ampicillin	0.25 g
Rifampicin	0.01 g
Pentachloronitrobenzene (PCNB)	0.1 g
Hymexazol	0.05 g

All antibiotics were either suspended or dissolved in 10 ml sterile distilled water, added to CMA after it was autoclaved at 121 °C for 15 minutes and cooled to 50 °C in a water bath and mixed thoroughly.

7. Rye Agar

Rye seed	60 g
Anachemia Agar	15 g
Sigma - Sucrose	20 g

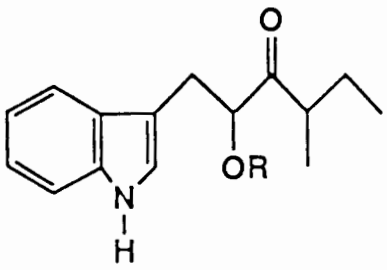
Rye seed in one liter of distilled water was soaked for 36 h at room temperature, autoclaved at 121 °C for 15 minutes, and strained through four layers of cheese cloth. The rye broth was topped to one liter with distilled water, mixed with agar and sucrose, and autoclaved again.

8. V8-Cholesterol Broth (Ayers and Lumsden, 1975)

V8 vegetable juice	200 ml
Calcium carbonate ($CaCO_3$)	2.5 g
Cholesterol (1.5 % solution in 95 % ethanol)	0.03 g

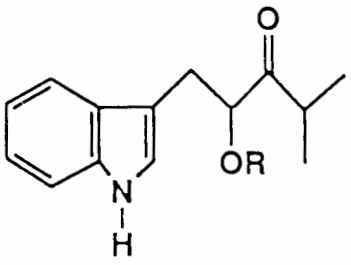
The mixture of V8 vegetable juice and $CaCO_3$ solution was clarified by centrifugation at 13,000 g for 30 minutes. The supernatant was topped to one liter with distilled water, added with cholesterol and autoclaved at 121 °C for 30 minutes.

APPENDIX VIII



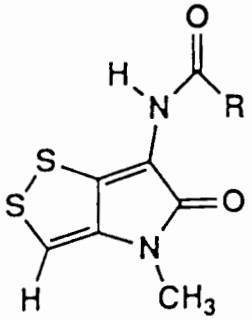
1: R = Ac

3: R = H



2: R = Ac

4: R = H



5: R = $CH_2CH_2CH_2CH_2CH_3$

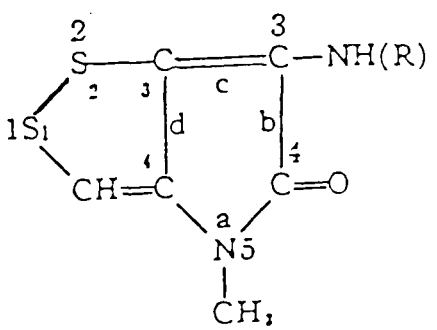
6: R = $CH_2CH_2CH_2CH(CH_3)_2$

7: R = $CH_2CH(CH_3)_2$

8: R = $CH_2CH_2CH_3$

Two groups of antibiotic, indole (1-4) and dithiolopyrrolone (5-8), isolated from *Xenorhabdus bovienii* A2 (Li *et al.*, 1995).

APPENDIX IX



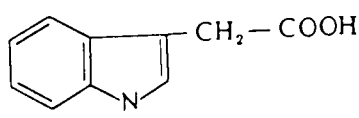
(a) R = CH_3CO-

(b) R = CH_3CH_2CO-

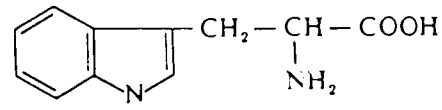
(c) R = $CH(CH_3)_2CO$-

The structure of (a) thiolutin and (b) aureothricin (Celmer and Solomons, 1955), and (c) isobutyropyrrothine (Bhate *et al.*, 1960), antibiotics isolated from *Streptomyces* species.

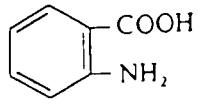
APPENDIX X



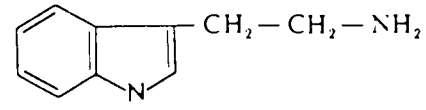
I



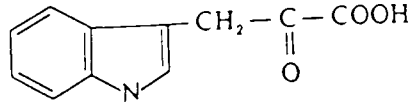
II



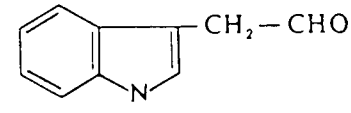
III



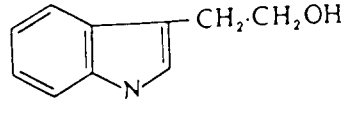
IV



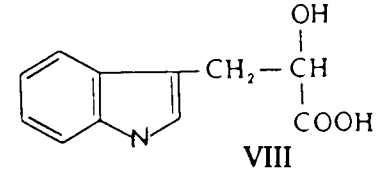
V



VI



VII



VIII

I	Indole-3-acetic acid (IAA)
II	Tryptophan α-amino-β-(indole-3)-propionic acid
III	Anthranilic acid
IV	Tryptamine β-(indole-3)-ethylamine
V	Indole-3-pyruvic acid
VI	Indole-3-acetaldehyde
VII	Tryptophol β-(indole-3)-ethanol
VIII	Indole-3-lactic acid

The natural auxins and related compounds (Audus, 1972).