Mining fungal secondary metabolites for novel antimicrobials

By

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Abstract

Evidence of antibiotic resistance evolving in various bacterial pathogens is accumulating, increasing the urgency to search for new antibiotics. Fighting antibiotic resistance requires a multi-pronged approach that includes studying the mechanism of resistance and mining for new antibiotics. Bacteria, such as *Streptomyces,* and fungi are an excellent source for secondary metabolites with antibacterial and other biological activities. The purpose of this study was to purify and characterize novel fungal isolates that produce antibiotics. Various geographical locations were selected for isolating fungi. A total of 570 fungal strains were isolated and 73 fungal strains showed antibacterial activities against an efflux-deficient strain of *Pseudomonas aeruginosa*. Six fungi were selected based on their antibacterial properties against Gram-negative (*P. aeruginosa* and *A. baumannii*) and Gram-positive (methicillin-resistant *Staphylococcus aureus*) bacteria for further analysis. One isolate labeled as 58-5, showed promising results as six unknown metabolites were detected by liquid chromatography-mass spectroscopy LC-MS. This isolate was selected for future experiments in order to identify and eventually produce these unknown metabolites. This work shows that fungi are an excellent source of novel antibiotics and thus can serve as an effective asset in our fight against antibiotic resistant bacteria.

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List of Abbreviations

ABC: ATP binding cassette

AMA: Aspergillomarasmine A

ATCC: American Type Culture Collection

ATP: Adenosine triphosphate

***benA*:** β-tubulin

bp: base pair

BIPM: Biapenem

***caM*:** calmodulin

CDC: Centers for disease control

CTAP: Cetyltrimethyl ammonium bromide

DNA: deoxyribonucleic acid

dNTPs: Deoxynucleoside triphosphates

EDTA: Ethylenediaminetetraacetic acid

EPI: Efflux pump inhibitor

EtOH: Ethanol

FDA: Food and Drug Administration

HIV: Human Immunodeficiency Virus

HMGR: 3-hydroxy-3-methyl-glutaryl-CoA reductase

HPLC: High Performance Liquid Chromatography

IMP: Imipenem-resistant *Pseudomonas*

ITS: Internal Transcribed Spacer

LC-MS: Liquid Chromatography Mass Spectroscopy

MATE: Multidrug and toxic efflux

MBL: Metallo-β-lactamase

MDR: Multi Drug Resistant

MDRP: Multi Drug Resistant *Pseudomonas*

ME: Malt Extract

MF: major facilitator

MIC: Minimum Inhibitory Concentrations

MRSA: Methicillin-resistant *Staphylococcus aureus*

NAG: *N*-acetylglucosamine

NAM: *N*-acetylmuramic acid

NCBI: National Center for Biotechnology Information

NDM-1: New Delhi metallo-β-lactamase

NMR: Nuclear Magnetic Resonance

OMP: Outer Membrane Protein

PBP: Penicillin Binding Protein

PCR: Polymerase Chain Reaction

RND: Resistance-nodulation-division

rRNA: Ribosomal ribonucleic acid

SM: Secondary Metabolite

SMR: Small multidrug resistance

***tef1*:** Translation elongation factor 1

UHPLC: Ultra High Performance Liquid Chromatography

VIM: Verona integrin-encoded metallo-β-lactamase

Chapter 1: Literature Review

1.1 A brief history of antibiotic

In 1890, Paul Vuillemin, used the word antibiosis to describe the biological competition between microorganisms (for example, fungi vs bacteria; bacteria vs protozoa) (1,2). Since that time, the word antibiotic was introduced to describe the secondary metabolites produced by bacteria and fungi that have either growth inhibitory or killing activities against microbes (1,2). In the Middle Ages, the miasma theory that means pollution, or contaminated air, was used to explain the causes of many health issues (3). In 1546, Girolamo Fracastoro, suggested that an unknown and a very small particles are responsible for certain diseases (3).

Scientists have been working hard for years to determine that bacteria are the small particles that responsible for causing certain diseases (3). The Hungarian physician Ignaz Semmelweis, believed that it is very important to keep hospitals sterilized and clean. He also was forcing other medical specialists to wash their hands with antiseptic agents before they examine patients (4). The Scottish operator Sir Joseph Lister, treated surgery wounds with phenol (carbolic acid) solutions to avoid contamination (4,5).

Efforts by the German physician Robert Koch lead to the discovery of the causative relationship between microbes and infectious diseases (6-9). In 1882, Koch, Fischer and Gaffky, described and identified *Mycobacterium tuberculosis,* and in 1884 *Vibrio cholerae* as the causative agents for Tuberculosis and Cholera, respectively (10). Once the causes of infectious

diseases were gaining understanding, scientists started to think of strategies to fight microbes, and develop strategies to cure infected people. In 1893, the microbiologist Bartolomeo Gosio, reported on the first antibiotic from nature which was mycophenolic acid (11). This compound was produced by a fungus called *Penicillium glaucum* (now *P. brevicompactum*) (11,12). It was shown that this compound possessed antibacterial activity against *Bacillus anthracis*, and also had antiviral, antifungal, antitumor and anti-psoriasis effects (13). Mycophenolic acid was reisolated and detected in 1913 in the USA. The use of this compound as antibacterial was abandoned in clinical use due to its adverse effects (14).

Staphylococci, a summer vacation, and observations by Alexander Fleming are three elements that helped in the discovery of the most important antibiotic: discovery of penicillin from the fungus *Penicillium notatum*. In September 1928, after returning from his vacation, Fleming went back to his laboratory, and noticed an agar plate with some *Staphylococcus aureus* colonies he had left on his bench contaminated with *Penicillium notatum* (15). In 1945, penicillin became available to the public saving millions of people lives (16).

Penicillin discovery and its dramatic success as antibiotic, encouraged many scientists and researchers to discover other antibiotics from natural sources. Progress of antibiotic discovery continued and led to the discovery of many potent antibiotics such as cephalosporin, gramicidin D, streptomycin, tetracyclines, chloramphenicol, erythromycin, vancomycin, and rifamycins (17-31).

Today, many infections caused by microbes are totally or partially relieved with a variety of currently available antibiotics. Characterization and isolation of secondary metabolites from microbes such as bacteria and fungi could be fruitful for finding new antibiotics in order to save lives. Microbiologists and organic chemists have a great role in the discovery and development

of these promising antibiotics. The fight against antimicrobial resistance is a continuous struggle as natural selection will always favor drug resistant pathogens in clinical environments.

1.2 Fungi as a promising source of highly valuable secondary metabolites

Fighting antibiotic resistance can be accomplished by various methods such as elucidating the mechanism of microbial resistance. Understanding microbial resistance mechanisms may lead to new and effective procedures for treating infectious diseases. Fighting antibiotic resistance can also include the discovery new compounds with antimicrobial properties such as antibiotics and adjuvants (for antibiotics) from microbial sources.

Fungi are well known for their production of secondary metabolites during active cell growth (32). Secondary metabolites are known as heterogeneous low-molecular-weight metabolites that are not essential for the organisms growth (33). These secondary metabolites are considered to be important for fungi. These compounds can protect against environmental stress (pH, dry conditions, UV light etc.), competing microbes, and they can be used by fungi for communication (34). Sometimes, the produced metabolites have toxic effects on both human and plant crops (32). In contrast, some secondary metabolites are beneficial for humans and can be used in the treatment of various types of diseases including infectious diseases (33). Many fungi are known for their capability to produce a broad range of bioactive and currently clinically used metabolites, including antibiotics, immune suppressants, and cholesterol-lowering drugs (32). Scientists have explored fungal secondary metabolites more intensively since the exploration of penicillin (33).

As fungi are a potential source of antibiotics, they are also a promising source for other medically important secondary metabolites (35). The story of penicillin discovery by Fleming inspired many scientists who were studying cholesterol biosynthesis and its relationship in causing diseases. HMG-CoA reductase enzyme is the main enzyme that responsible for cholesterol synthesis (36). This enzyme must be targeted by a specific substance to control high cholesterol levels. In 1968, a group of researchers at Sankyo Research Laboratories (Tokyo, Japan), worked on a project that targeted the HMGR enzyme. Inspired by Fleming's success with molds, they explored fungi for metabolites with cholesterol lowering properties. In 1971 after screening high number of fungi, they found a sample of mold that showed great inhibitory activity. The isolated compound appeared to be citrinin. Citrinin inhibited HMG-CoA reductase, and lowered cholesterol levels in tested mice (36). However, they suspended the study because of citrinin toxicity to the kidneys. This discovery encouraged Scientists to find better active compounds in the future.

In 1972, the same group found another active culture broth of *Penicillium citrinum*. They were able to isolate ML-236B, referred as compactin (36,37). After several years of clinical and toxicological studies, they discontinued the development of compactin but as this compound was found to cause lymphoma in dogs (38).

Merck researchers continued work on compactin and were able to discover a new compound similar to compactin in chemical structure (36). The new compound was lovastatin (39). After several clinical trials, U.S. FDA approved lovastatin in 1987 as the first member of statins group. Since lovastatin had been commercialized, 6 statins, including 2 semi-synthetic statins (simvastatin and pravastatin) and 4 synthetic statins (fluvastatin, atorvastatin, rosuvastatin

and pitavastatin) have been introduced to the market (36). Today, statins are saving millions of people lives (36).

Sandoz, a Swiss pharmaceutical company, explored fungal secondary metabolites with antimicrobial, anticancer, and immunosuppression activity (40,41). In one of their soil samples collected from Norway, they isolated a fungus called *Tolypocladium inflatum* (40). Secondary metabolites extracted from this fungus showed weak antifungal effects, but no antibacterial properties (42). However, this isolate produced a compound that had unique immunosuppressive activity (40); the compound was cyclosporine A (40,43).

In 1983, cyclosporine A was approved for clinical use, and is the first choice treatment after organ transplants (44). Cyclosporine A successfully replaced the previously used medications such as azathioprine and corticosteroids, providing more safety and less side effects to the patients (45).

1.3 Novel fungal secondary metabolites and adjuvants to combat antibiotic resistance

Antibiotic resistance is rising to dangerously high levels (46,49). New resistance mechanisms are emerging and spreading, making the treatment of infections very challenging (47). Antibiotic / Antimicrobial resistance happens when an antibiotic is not able to control bacterial growth (48). The Centers for Disease Control and Prevention Department of Health and Human Services in the United States, claims that antibiotic resistance causes at least 2.8 million illnesses and 35,000 deaths in the USA each year (49).

Bacterial antibiotic resistance is the result of mutations that select for bacterial cell to survive in challenging environments such as the presence of antibiotics (50). Some mutations give the bacteria the ability to produce enzymes that destroy antimicrobials, whereas other mutations change the target that the antibiotic works on (51). Other mutations prevent the antibiotic to pass through the cell wall (permeability), and others promote and the activation of pump mechanisms that export the antibiotic outside the cell (efflux) (51). Bacteria can acquire resistance genes from other bacteria via transformation and conjugation that results in the transfer of genetic material, including genes encoding resistance from one bacterium to another (52,53).

Antimicrobial resistance has a negative impact on both, people's health and the economy (54). This issue arose from misuse and overuse of antibiotics resulting in the selection of bacterial resistance mechanisms (55), leading to multidrug resistant bacterial cells making treatment challenging with current available antibiotics (56). Previous studies suggest without any actions, microbial resistance will kill more than 10 million people by 2050, a number that surpasses the deaths caused by cancer (57). Smart and effective treatment strategies of infectious diseases are therefore important. Strategies such as combining antibiotic/antibiotic, antibiotic/adjuvant, and antibiotic/antivirulence compounds are promising (58).

Strategies for more effective antibiotic treatments could include the combination of antibiotics ("multidrug cocktails") (58). For example, combining two antibiotics at a certain dose might overcome the bacterial resistance mechanisms issue plus it would be more difficult to evolve mechanisms that alter two targets within a cell in order to escape the applied drugs. Antibiotic combinations aim to attack different bacterial targets at the same time in order to enhance the antimicrobial effect of the drugs, and this can occur by: (i) inhibition of antibiotic

resistance enzymes; (ii) enhancement of the uptake of the antibiotic through the bacterial membrane by increasing the permeability of cell membrane; (iii) direct blocking of efflux pumps (59).

Combinations that include aminoglycosides plus ampicillin/sulbactam, and carbapenem with colistin, and colistin with rifampin have been successful against multi-drug resistant *Acinetobacter spp* (60-62). However, the combination strategy of these antibiotics has gradually led to the emergence of multi-drug resistant pathogens, and also the emergence of new antibacterial resistant mechanisms by the bacteria. In addition, multi-antibiotic combinations could increase the chance of unwanted side effects among infected people. Therefore, an alternative for treatment of infectious diseases with antibiotic combination therapies is to find adjuvants of existing antibiotics.

Potentiators of antibiotic activity are sometimes referred to as antibiotic adjuvants. Antibiotic adjuvants are non-antibiotic compounds that enhance antibiotic activity by protecting antibiotics from various resistance mechanisms. Adjuvants can via a variety of different mechanisms reactivate an old life-saving antibiotic, and make them useful and robust again (63,64). β-lactamase inhibitor adjuvants are a good example of successful and promising adjuvants. The combination of β-lactam antibiotic with β-lactamase inhibitor adjuvant is the most successful and clinically used combination strategy to date. Since the discovery of β-lactam antibiotics, countless lives have been saved, and they continue to be one of the fundamental drugs for treating bacterial infections (64-70).

Resistance to β-lactam antibiotics can be through various mechanisms: (1) Production of β-lactamase enzymes. Bacterial β-lactamases are enzymes that inhibit the effect of β-lactam antibiotics such as penicillins, monobactams, and carbapenems by attacking their β-lactam rings.

Hydrolysis of β-lactam ring results in a microbiologically inactive antibiotic. (2) Lowering the affinity of b-lactam antibiotic to the target site by mutations in the penicillin binding protein (PBP) that are located in bacterial membranes (71). (3) Reducing the expression of outer membrane proteins (OMPs) is another example of a resistance mechanism. β-lactams must diffuse through the porin channels in the outer membrane of Gram-negative bacterial cell walls to access PBPs on the inner plasma membrane in order to give the antibacterial activity. (4) Efflux pumps, are able to expel a high range of compounds out of the cell. These types of pumps are a significant determinant of multidrug resistance in many Gram-negative pathogens, such as *P. aeruginosa* and *Acinetobacter* spp (72).

In response to these antibiotic resistance mechanisms, two strategies have been done to maintain the usefulness of β-lactam antibiotics: (i) discover or design β-lactam antibiotics that are able to bypass bacterial enzymatic inactivation conferred by β-lactamases, or (ii) inhibit β-lactamases allowing the β-lactams to reach the PBP, the target site of β-lactam antibiotics (73).

The β-lactamase inhibitor adjuvant can address any one of the issues raised above by boosting the activity of the antibiotic by inhibiting the function of β-lactamase. Thus, the adjuvant can restore the activity of the β-lactam antibiotic in β-lactamase producing pathogens (74).

1.4 β-Lactamase inhibitors supporting β-lactam antibiotic activity

Clavulanic acid, sulbactam, and tazobactam (75). Clavulanic acid was the first adjuvant introduced into clinical medicine that inhibits β-lactamase. It was isolated from a Gram-positive bacterium called *Streptomyces clavuligerus* in 1970 (76). Clavulanate showed little antimicrobial

activity alone, but when combined with amoxicillin, it significantly lowered the amoxicillin minimum inhibitory concentrations (MICs) against *S. aureus*, *K. pneumoniae*, *Proteus mirabilis*, and *Escherichia coli* (77). Sulbactam and tazobactam that were later developed by the pharmaceutical companies as synthetic compounds in 1978 and 1980, respectively (78,79). All three β-lactamase inhibitor compounds share structural similarity with penicillin; are effective against many susceptible organisms expressing class A β-lactamases (80-82).

Avibactam is a novel non β-lactam β-lactamase inhibitor adjuvant that inhibits both class A and class C (and some class D) enzymes (83,84). It is currently in clinical development in combination with ceftazidime and ceftaroline fosamil and with the monobactam aztreonam as an alternative option for the treatment of infections caused by multidrug-resistant *P. aeruginosa* and *Enterobacteriaceae* spp. (85,86).

1.5 Phthalic acid derivatives, Succinic acid derivatives, Aspergillomarasmine A, and Compounds 1 and 2 Polyketides.

Metallo-β-lactamases family (MBLs) belongs to class B β-lactamase that can hydrolyze and inactivate all β-lactam antibiotics (87). The β-lactam antibiotic deactivation process that is carried out by MBLs is achieved by the use of zinc atoms that activate a nucleophilic water molecule that opens the lactam ring (87). Various types of metallo-β-lactamases such as IMP-type carbapenemases, verona integrin-encoded metallo-β-lactamase (VIM), and New Delhi metallo-β-lactamase (NDM-1) have been reported (88,89). Carbapenems play an important role in the clinical field because they are highly effective against both Gram-positive and Gram-negative bacteria including *Pseudomonas aeruginosa*. Thus, MBL-producing pathogens are a

critical problem in the clinical field. In addition, multi-drug resistance *P. aeruginosa* strains (MDRP) are a significant issue because of their resistance against aminoglycosides, carbapenems and quinolones, which are normally effective against Gram-negative bacteria (90). Therefore, there is a need for inhibitors of IMP-1 metallo-β-lactamase that could protect broad spectrum antibiotics. In general, chemists are interested in developing MBL inhibitors (91-92).

Aspergillomarasmine A (AMA) is a fungal metabolite that was extracted and purified from a fungus called *Aspergillus versicolor* (93). Many studies revealed that this metabolite can be considered to be an antibiotic adjuvant as it restored the activity of meropenem antibiotic, and made meropenem–resistant bacteria sensitive again to this antibiotic. *In vivo* studies showed that AMA restored the clinical efficacy of meropenem antibiotic. In a mouse model of NDM-1-positive *K. pneumoniae* infection, a single dose of the combination of meropenem (10 mg/kg of body weight) and AMA (30 mg/kg) led to 95% survival after 5 days' post infection, whereas the use of meropenem or AMA alone at the same concentrations resulted in 0% survival (94). AMA has successfully passed phase I and phase II clinical trials, and now it is in the third clinical trial (94,95).

1.6 Pump inhibitors promoting antimicrobial activity of antibiotics against resistant bacteria

In 1980, efflux resistant mechanism was first described following the identification of tetracycline resistance in *E. coli* (96,97). The main role of such pumps is to protect bacteria from toxins and antibiotics (98,99). Among the prokaryotes there are five major families of efflux systems: MF (major facilitator), MATE (multidrug and toxic efflux), RND (resistance-

nodulation-division), SMR (small multidrug resistance) and ABC (ATP binding cassette). All these systems utilize the proton motive force as an energy source, apart from the ABC family, which utilizes ATP hydrolysis to drive the export of substrates (100,101). Efflux pump inhibition is a strategy that could re-establish the activity of current antibiotics against resistant bacteria (102). Searching for new potent antibiotics with efflux pump inhibition properties, remains the main strategy against efflux mediated drug resistance (103). Combining an efflux pump inhibitor (EPI) with an antibiotic agent, could prevent the expulsion of the antibiotic and increase its concentration inside the bacterial cells (104,105). Therefore, EPIs are expected to increase the potency of currently available antibiotics and to expand their spectrum of activity.

1.7 Boronic acid derivatives (inhibits pump: NorA, target: *S. aureus*)

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are of particular concern among resistant microorganisms because they are responsible for hospital acquired infections (106,107). NorA-overexpressing strains have been shown to be the most common among MRSA strains (108). Its overexpression confers resistance to a wide range compounds such as hydrophilic fluoroquinolones (ciprofloxacin and norfloxacin but not lipophilic ones), various biocides (acriflavine, cetrimide, and benzalkonium chloride), and dyes (ethidium bromide) (109,110). In recent years, several NorA inhibitors have been identified (111). The alkaloid reserpine, the first natural identified NorA EPI, is commonly used as a control in pump inhibitory assays. Reserpine showed neurotoxicity side effect, therefore it is not clinically used (112).

Various researchers have focused on the synthesis and physicochemical study of various heterocyclic compounds bearing boronic acid or boronic ester functions. Boron-containing compounds (BCCs), have become attractive molecules in drug development. Several studies claimed that adding boron atoms to the drugs will increase the effectiveness of those drugs (113). Various studies based on preliminary toxicological results suggested that BCCs can be considered potential therapeutic agents in the development of new drugs (114-122).

1.8 Capsaicin (inhibits pump: NorA, target: *S. aureus*)

Capsaicin is the major ingredient of hot chilli, a member of the genus Capsicum. It has a wide range of biological activities in humans, affecting the nervous, cardiovascular and digestive systems as well as finding use as an analgesic (123-125). Although possibly detrimental to the human gastric mucosa, capsaicin is also bactericidal to *Helicobacter pylori* (126).

Capsaicin in combination with ciprofloxacin was tested for activity against *S. aureus* (NorA overproducing strain). It has significantly reduced the MIC of ciprofloxacin. The most significant finding was the ability of capsaicin to reduce the intracellular invasion of *S. aureus* (NorA overproducing) in macrophage (127).

1.9 Phenylalanine-arginine ß-naphthylamide (inhibits pump: RND, targets: various Gram-negative bacteria)

The resistance nodulation division (RND) superfamily of efflux pumps are the primary resistance mechanism in *P. aeruginosa*. They are responsible for extruding a broad spectrum of

antimicrobial compounds (128). The major RND efflux pumps of high clinical importance in *P. aeruginosa* are MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM, and they differ in their expression patterns and substrate specificities (129). Antibiotic substrates for efflux pumps include β-lactams, fluoroquinolones, and aminoglycosides (130). MexAB-OprM is the constitutively expressed primary efflux pump in *P. aeruginosa* and responsible for the majority of efflux activity (131,132). Deletion of this pump makes *P. aeruginosa* highly susceptible to β-lactam antibiotics (even those strains overexpressing AmpC), AmpC β-lactamases are clinically important cephalosporinases encoded on the chromosomes of many of the Enterobacteriaceae and a few other organisms, where they mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and β-lactamase inhibitor-β-lactam combinations. Its overexpression increases antibiotic resistance (133,134).

A peptidomimetic compound, phenylalanine-arginine β-naphthylamide (PAβN), is one of the best studied EPIs that has as a broad-spectrum efflux pump inhibitor activity (135). This compound has the ability to reduce fluoroquinolone resistance in *P. aeruginosa*. In addition to its efflux activity, PAβN was reported to permeabilize membranes at concentrations ≥16 mg/mL, but only in *P. aeruginosa* mutants deficient in the MexAB-OprM efflux pump (136). Treatment with PAβN permeabilized the outer membrane of all *P. aeruginosa* strains tested, causing the release of AmpC β-lactamase into the culture supernatants. Also, PAβN sensitized cells to vancomycin, an antimicrobial that is normally ineffective against *P. aeruginosa* because it cannot cross the outer membrane (137). The reduction of the intrinsic resistance of *P. aeruginosa* to β-lactam antibiotics has also been documented (137).

1.10 Virulence factors as targets

Antivirulence drugs target virulence factors that are expressed during infection process. These virulence factors are essential for bacterial pathogenesis. Certain virulence factors in pathogenic bacteria, such as toxins, and host cell destroying enzymes are mostly absent from nonpathogenic species (138). In the absence of the virulence factors, bacteria will be unable cause a pathological infection in their hosts, and the host immune system can work more effectively in the absence of virulence factors. In addition, the local microbial flora are more likely can outcompete pathogens in the absence of such factors. Targeting virulence factors can be effective, for example many bacterial vaccines are directed in whole or in part promote raising antibodies to neutralize toxins or other virulence factors. An attractive feature of using drugs directed against virulence factors are less likely to trigger resistance phenotypes as they do not interfere with pathways that are essential for cell viability. Also these types of drugs may not disrupt the normal flora as its members usually lack virulence factors (139).

1:11 Concluding remarks

To effectively fight microbial antibiotic resistance, researchers and medical practitioners should place a lot of effort to extend the effectiveness of the currently available antibiotics. There are many strategies to do this. First: restricting the quantity of antibiotics that are applied in agricultural systems in order to reduce the selection pressure on mutations that promote antibiotic resistance. Second: cessation of unnecessary antibiotic prescriptions that are given for non-bacterial diseases/infections such as the flu. Third: educating the public about the correct way of using antibiotics, so they have to finish their entire antibiotic treatment course even when

they are feeling better. Fourth: researchers must focus on discovering new antibiotics, and most importantly mining for adjuvants, and antivirulence factors compounds from biological sources that could revitalize the current antibiotics that are prone to failure due to antibiotic resistance.

Employing adjuvants will enable us to use extant antibiotics on microbes that have developed resistance. As plants, terrestrial microbes, and marine microbes are promising sources of antibiotics, they could also be a great source of antibiotic adjuvants. Bioprospecting for novel compounds is therefore an important component of a multipronged approach for mitigating antibiotic resistance in microorganism.

The objectives of this work is to examine the potential of fungal strains isolated from various niches (soil, beaches, insects etc.) to produce antimicrobial compounds. The long term goal is to find new compounds that can negate the increasing problems associated with the evolution of antibiotic resistance in bacteria.

Chapter 2: Materials and methods

2.1 Collection of samples and cultivation of fungi

Soil samples were collected from three different locations in Manitoba as a potential source of fungal isolates. These include Grand beach at Lake Winnipeg (50°33'37.7"N 96°37'24.5"W), Falcon beach at Falcon Lake (49°41'06.3"N 95°19'20.1"W), and Aurora Farm (49°43'47.0"N 97°10'48.1"W). Fungal isolates were also recovered from two fruit flies. Each collected sample was given a number, placed and stored in plastic bags. Each isolated fungus strain was given an ID number (Table 2.1).

Table 2.1: Fungi isolated from various sources in Manitoba

Sampling sources/locations	Number of samples collected	ID of isolated fungal strains
Falcon beach, Falcon Lake	6	51-1
		51-2
		51-3
		51-4
		51-5
		51-6
		51-7
		51-8
		51-9
		51-10
		51-11
		51-12
		51-13
		52-1
		52-2
		52-3
		52-4
		52-5
		52-6
		52-7
		52-8
		52-9
		52-10
		52-11

		52-12
		52-13
		52-14
		52-15
		53-1
		53-2
		55-1
		56-1
		57-3
		57-5
		58-5
		58-32
		58-35
Grand beach, Lake Winnipeg	3	38-1
		38-2
		40-3
		40-5
		40-6
		40-7
		40-9
		40-10
		41-7
		41-9
		41-10
		41-12
		41-13
		41-14
		41-15
Aurora farm, Winnipeg	3	42-1
		42-2
		42-3
		42-9
		44-2
		44-10
		44-12
		44-13
		44-14
		44-18
		44-22
		46-2
		46-6
Two fruit flies, Winnipeg	2	90-4
		100-1
		100-2
		100-3
		100-11
		100-14
		100-15
		100-16

In order to isolate individual fungal colonies, 100 mg of soil was added to a sterile 50 mL conical centrifuge tube (ThermoFisher Scientific, Waltham Massachusetts, USA) containing 10 mL of ddH_2O. The soil suspension was incubated at room temperature for 1 hour. Malt extract agar [(MEA) OXOID, Nepean, ON, Canada] was prepared and supplemented with rose Bengal (Hartman-Leddon company, Philadelphia, Pennsylvania, USA) solution (3.3 g/L) to slow down the growth of fungi and to make the isolation of fungal colonies much easier (140) (rose Bengal at a final concentration of 0.05 g/L of MEA) and chloramphenicol antibiotic to inhibit the growth of bacteria (Sigma-Aldrich Canada, Oakville, ON, Canada) [per liter: 30 g ME, 20 g agar, 500 µL rose Bengal solution, 1 mL of 3 mg/100 mL chloramphenicol)]. After incubation, 100 µL of the suspension was added to the ME/rose Bengal plates and spread with the triangular glass rod. Duplicate plates were made and sealed with parafilm, and incubated for 3 to 5 days at 22ºC and 37ºC, respectively. Once fungal colonies started growing, individual colonies were picked up with wooden sterile inoculation sticks (VWR) and transferred to fresh MEA plates and incubated at 22ºC for 5 to 7 days. Plates were checked for contamination and pure plates were kept while the contaminated ones were eliminated. Similar conditions were applied to the insect samples. Except that the insects were washed three times with ddH_2O to dislodge microorganisms that are on the surface of the insects. The fruit flies were crushed with wooden sterile inoculation sticks and added to a sterile 50 mL conical centrifuge tube containing 10 mL of ddH_2O.

2.2 Plug agar assay and bacterial strains

Each isolated fungus was tested for antibacterial activity against the sensitive *Pseudomonas aeruginosa* PA0750 strain [lacks five major RND efflux pumps (141)]. PA0750 cultures were routinely cultured in lysogeny broth (LB) (Becton, Dickinson and company,

Sparks, Maryland, USA) (20g per 1L) and incubated overnight at 37ºC shaker incubator at 250 rpm. Bacterial cells were then diluted in saline (0.85% NaCl) (Fisher Scientific, Ottawa, ON, Canada) (0.85% NaCl concentration) and cell numbers were adjusted to a density of 10^8 CFU/mL using a 0.5 McFarland turbidity standard (BioMerieux, Durham, NC, USA). Diluted bacterial cultures were spread on LB agar (35g per 1L) plates using cotton swabs. A circular plug of fungal agar culture was generated by cutting a circle using a surgical knife (World precision instruments, Sarasota, FL, USA) and the plug was placed on the LB agar plate containing bacteria. The plate was incubated for 16 to 20 hours at 37ºC. Fungi that showed zone of inhibition (antibacterial activity) are kept and maintained in triplicates on MEA slants, and these were stored at 4ºC for further analysis. Fungi with antibacterial activity are also challenged against Gram negative wild type strains such as *P. aeruginosa* PA01, and *Acinetobacter baumannii* ATCC17978 bacteria, clinical isolates of multidrug resistant (MDR) *A. baumannii* AB030, and *P. aeruginosa* PA82, and the Gram positive bacteria methicillin-resistant *Staphylococcus aureus* (MRSA) (see Table 2.2 for details on the bacterial strains).

Table 2.2: Bacterial strains used in the study

Species name	Strain number	Properties	Reference
Pseudomonas aeruginosa	PA0750	Antibiotic sensitive strain (lacks five RND efflux pumps)	141
Staphylococcus aureus	MRSA	Methicillin-resistant *Staphylococcus aureus*	Kumar lab collection
Pseudomonas aeruginosa	PA01	Wild type	142
Acinetobacter baumannii	ATCC17978	Wild type	ATCC
Acinetobacter baumannii	AB030	Clinical isolate and multi drug resistant	143
Pseudomonas aeruginosa	PA82	Clinical isolate and multi drug resistant	144

2.3 Extraction of nucleic acids

Nucleic acids were extracted from fungal mycelium as previously described by Hausner et al. (1992) and Abboud et al. (2018) with modification as described below (148). The mycelia of fungi cultured on solid MEA media were harvested by scraping the mycelia from the ME plates. The mycelium was weighed and ground up with acid washed sand and in the presence of 2X Cetyltrimethyl ammonium bromide (CTAB) buffer [2% CTAB (Fisher Scientific), 100 mM Tris-HCl, pH8 (Fisher Scientific), 20 mM Ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific), 1.4 M NaCl]. The extraction mixture was composed according to the following ratio: 1 g of mycelia to 1.5 g of sand per 2 mL of 2X CTAB buffer. The mixture was ground with a pestle for 5 to 10 minutes until it resembled a slurry. The slurry was transferred into a 50 mL sterile conical centrifuge tube and incubated for 1 hour at 65ºC water bath with gentle mixing every 20 minutes. The mixture was then cooled for 1 minute at 15°C. An equal volume of chloroform (Fisher Scientific) was added to the mixture, after mixing the tube was centrifuged at 3100 RPM/1510 RCF for 10 minutes. Following centrifugation, the top aqueous layer was transferred to a new 50 mL sterile conical tube and extracted a second time by adding an equal volume of chloroform. The tube was centrifuged again at 3100 RPM/1510 RCF for 10 minutes, thereafter the aqueous layer was transferred to a new 50 mL sterile conical tube. The chloroform extraction process was repeated until the aqueous layer became clear. Finally, the DNA was precipitated by adding three volumes of 95% ethanol (EtOH) to the recovered aqueous layer (Fisher Scientific) the mixture was mixed gently and placed at -20ºC for 1 to 5 hours. To pellet the nucleic acids, the mixture was centrifuged at 3500 rpm for 15 minutes. Finally, the EtOH was decanted and 1 mL of 70% EtOH is added to wash the DNA pellet and remove NaCl; the tube was briefly centrifuged at 3100 RPM/ 1510 RCF for 5 minutes. Then, the EtOH was completely

removed from the tube by decanting EtOH. The tube was kept open to allow the EtOH to completely evaporate. To each dried pellet 300 µL of sterilized ddH_2O was added in order dissolve the pellet; the DNA samples were stored at -20ºC in sterile 1.5 ml microcentrifuge tubes.

2.4 Molecular markers used for fungal identification

Internal transcribed rDNA spacer (ITS), β-tubulin, calmodulin, and elongation factors are molecular markers that were amplified for genetic identification using standard Polymerase chain reaction protocols [PCR (see Table 2.3 for the PCR protocols for each primer combination)]. The PCR reactions were set up as follows in 50 µL volumes: 5 µL of Taq Buffer (GeneDirX, Inc., Taoyuan City, Taiwan), 1 µL of 10 mM dNTPs (final concentration is 0.2 mM) (Invitrogen, Burlington, ON, Canada), 1 µL of 10 µM forward primer (final concentration is 0.2 µM) (Alpha DNA, Montreal, Quebec, Canada), 1 µL of 10 µM reverse primer (Alpha DNA), 0.5 µL of Taq DNA (GeneDirX), 1 µL of template DNA, and nuclease free water (Invitrogen) to make the volume to 50 µL. The primers (see Table 2 for more information) used for amplification were as follows: ITS1 & ITS4 for the ITS), Bt2a & Bt2b for β-tubulin, CMD5 & CMD6 for calmodulin, and EF1 & EF3 for the translation elongation factor 1-alpha. All PCR amplicons were analyzed by gel electrophoresis through 1% agarose gels in TBE buffer (89 mM Tris-borate, 10 mM EDTA, pH 8.0) and DNA bands were visualized by staining with ethidium bromide (0.5 µg/mL). PCR amplicons were purified using the PCR purification kit (QIAGEN), and the PCR products were sent for Sanger sequencing to the Manitoba Institute of Cell Biology at the University of Manitoba. The sequences were examined in GeneDoc (149) and further analyzed using BLASTn in National Center for Biotechnology Information (NCBI) to find

related sequences (150). The following databases were also applied: International Subcommision on *Hypocrea* and *Trichoderma* (ISHT) (URL: http://www.isth.info/tools/blast/index) for *Trichoderma* spp. identification, and Westerdijk Fungal Biodiversity Institute database (URL: http://www.wi.knaw.nl/fusarium/**)** for *Fusarium* spp. identification (151,152).

Table 2.3: PCR protocols used in fungal identification

Locus	Primers	Primers Sequence 5'-3'	Direction	PCR protocols	Reference
Internal Transcribed Spacer (ITS) region of rRNA [~ 450-800 bp]	ITS1 ITS4	CTT GGT CAT TTA GAC GAA GTA A TCC TCC GCT TAT TGA TAT GC	Forward Reverse	95ºC – 3 min 95ºC – 30 s 58ºC – 45 s 72ºC – 2 min Repeat 2-4 40 cycles 72ºC – 8 min 4ºC on hold	144
β-tubulin (*BenA*) [~500 bp]	Bt2a Bt2b	GGT AAC CAA ATC GGT GCT GCT TTC ACC CTC AGT GTA GTG ACC CTT GGC	Forward Reverse	95ºC – 5 min 95ºC – 45 s 58ºC – 45 s 72ºC – 1 min Repeat 2-4 35 cycles 72ºC – 7 min 4ºC on hold	145
Calmodulin (*CaM*) [~580 bp]	CMD5 CMD6	CCG AGT ACA AGG AGG CCT TC CCG ATA GAG GTC ATA ACG TGG	Forward Reverse	94ºC – 1 min 94ºC – 1 min 55ºC – 30 s 72ºC – 1.5 min Repeat 2-4 35 cycles 72ºC – 10 min 4ºC on hold	146
Translation elongation factor 1-alpha (*tef1*) [~600 bp]	EF1-1018F EF1-1620R	GAY TTC ATC AAG AAC ATG AT GAC GTT GAA DCC RAC RTT GT	Forward Reverse	94ºC – 5 min 94ºC – 30 s 42ºC – 30 s 72ºC – 1 min Repeat 2-4 30 cycles 72ºC – 5 min 4ºC on hold	147

2.5 Fungal secondary metabolites extraction

Two types of broth media were used for the solid state fermentation process. The fungal media used in this study were ME media (15 g malt extract per 1 L of dd H_2O) and YESD media [20 g soy peptone (Difco Laboratories, Detroit, MI, USA), 20 g dextrose (Difco Laboratories), 5 g yeast extract (Becton), in 1 L dd H_2O). Based on previous reports and initial efforts in this study two media (YESD and ME) were selected as they yielded the best growth. Fungi were inoculated in 500 ml sterilized broth media and incubated in a shaker incubator at 25ºC, at 130 rpm for 3 weeks. Following the incubation, the media were filtered using vacuum filtration with 0.2 µm pore size Millipore filter (VWR™, Edmonton, AB, Canada). The filtered media was transferred to a l L separatory funnel and acidified by adding 5 mL of concentrated HCl per 500 mL of filtered media. In a fume hood, 500 mL of ethyl acetate was added to the acidified media and mixed well. For the extraction purpose, ethyl acetate solvent was used because it is immiscible with aqueous solvent (the media), has medium polarity, and secondary metabolites can be easily solubilized in the organic solvent (ethyl acetate) (153). The spent media was extracted 3 times with an equal volume of ethyl acetate. The organic solution was transferred to a clean bottle and anhydrous sodium sulfate was added (100 gm of anhydrous sodium sulfate to 500 mL of organic solution). The organic solution was filtered and transferred to an evaporation flask for vacuum evaporation using a rotary evaporator. After the organic solvent evaporated, the residue of crude extract was redissolved in 2-3 ml of HPLC grade methanol (Fisher Scientific).

The crude extract dissolved in methanol was transferred to an evaporation vial; this was dried again for long term storage. To confirm antibacterial activity of the extracted fungal metabolites a plate of LB inoculated with an overnight culture of PA0750 was prepared, 10% of

the crude extract was dissolved in 1 mL dimethylsulfoxide (DMSO), and 10 µL of the dissolved crude was placed on a sterilized filter disc. The disc was placed on top of the bacterial culture and the plates were assayed for the occurrence of a zone of inhibition.

2.6 High-performance liquid chromatography (HPLC) analysis

HPLC analysis was performed at the Chemistry Department of the University of Manitoba, courtesy of Mona Abdel-Hameed (154). The following protocol was performed: the dried crude extract was weighted and dissolved in methanol to a final concentration 0.5 mg of crude extract/mL. HPLC analysis was carried out on a Waters HPLC Separations Module 2695, combined with a PDA Detector Model 2996. The column was a µBondapak® Waters C18 (3.9 X 300 mm) with a column particle diameter of 15-20 µm with 125 Å pores. The flow rate was 1 mL/minute. The eluent was monitored continuously at 210-600 nm, and HPLC traces were displayed at 254 nm. The gradient was held at 20 % methanol to 80 % water in 0.075 % aqueous trifluoracetic acid for 10 minutes then the percent of methanol was linearly increased up to 80 % and held at that composition for 20 minutes followed by a linear gradient back to 20 % methanol for 10 minutes and held there for 10 minutes. The total run time was 60 minutes.

2.7 Sample preparation for liquid chromatography–mass spectrometry (LC-MS)

Dry crude spent media extracts were prepared (see further above) and sent to Dr. Russell Kerr, Department of Chemistry, University of Prince Edward Island (PEI) for LC-MS analysis. The following protocol was performed: Thermo Accela UHPLC Pump, Thermo Velos HRMS fitted with an ESI source, and Thermo PDA. A Phenomenex Synergi Polar-RP 80 Å column (150 × 4.60 mm, 4 µm, was used with a mobile-phase flow rate of 0.5 mL/min and injection

volume of 10 μL (all samples were prepared in CH_3OH). The following elution method was used [A = H_2O (0.1% formic acid), B = CH3CN (0.1% formic acid)]: 5% B from 0.0 to 0.2 min, linear gradient from 5% B at 0.2 min to 99% B at 4.8 min, 99% B from 4.8 to 8.0 min, linear gradient from 99% B at 8.0 min to 5% B at 8.5 min and 5% B from 8.5 to 10.0 min. The following HRMS parameters were used: positive ionization mode, mass resolution of 30,000, mass range of m/z 190 to 2,000, spray voltage of 2.0 kV, capillary temperature of 320 °C, S-lens RF voltage of 60.0%, maximum injection time of 10 ms, and 1 microscan. The system was controlled by Thermo Xcalibur software modules (155).

Chapter 3: Results

3.1 Screening fungi for antibacterial properties

For this study, soil samples from various geographical locations in Manitoba were collected to isolate fungi and screened for antibacterial activity. In addition, one fungal isolate purified from *Drosophila* sp. from Winnipeg was also analyzed. Out of a total number of 570 fungal isolates purified, 73 showed antibacterial activity against the antibiotic sensitive (efflux deficient) strain *P. aeruginosa* PA0750 (see Table 3.1). These 73 fungal strains were then tested against Gram negative bacteria *P. aeruginosa* PA01 (wild type), *A. baumannii* ATCC17978 (wild type), and clinical isolates of multidrug resistant (MDR) *A. baumannii* AB030, and *P. aeruginosa* PA82, and the Gram positive bacteria such as the methicillin-resistant *Staphylococcus aureus* MRSA.

The antibacterial effect of these fungi were varied, as some fungi were effective only against Gram negative bacteria, while others only showed activity against the Gram positive MRSA. One of the isolates, 40-10, showed antibacterial activity against all bacterial strains with more than a 20 mm inhibition zone except for ATCC17978 which showed a 10 mm inhibition zone (Table 3.1) based on agar plug assays. The 40-10 isolate based on ITS rDNA and β-tubulin gene sequences appears to be *Penicillium griseofulvum*. *P. griseofulvum* is well known as a mycotoxins producer including compounds such as patulin, cyclopiazonic acid, roquefortine C (156,157).

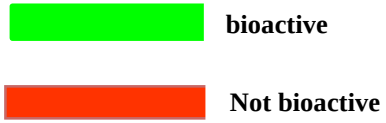


Table 3.1: List of fungal isolates (from a screen of 570 isolates) that exhibited activity against at least one of the bacterial strains/species tested. Zones of inhibition (based on agar plug assays) are indicated in mm

Sample ID	PA0750	MRSA	PA01	ATCC17978	AB030	PA82
38-1	15 mm					
38-2	25 mm	15 mm				
40-3	10 mm					
40-5	10 mm					
40-6	10 mm					
40-7	10 mm					
40-9	10 mm					
40-10	20 mm	22 mm	17 mm	10 mm	30 mm	20 mm
41-7	10 mm					
41-9	10 mm					
41-10	10 mm					
41-12	10 mm					
41-13	10 mm					
41-14	10 mm					
41-15	10 mm					
42-1	10 mm					
42-2	10 mm					
42-3	10 mm					
42-9	10 mm					
44-2	10 mm					
44-10	15 mm					
44-12	10 mm					

Sample ID	PA0750	MRSA	PA01	ATCC17978	AB030	PA82
44-13	10 mm					
44-14	10 mm					
44-18	15 mm					
44-22	15 mm					
46-2	15 mm					
46-6	10 mm					
51-1	10 mm	20 mm				
51-2	13 mm	20 mm				
51-3	10 mm	20 mm				
51-4	15 mm	20 mm				
51-5	15 mm	20 mm				
51-6	10 mm	20 mm				
51-7	12 mm	20 mm				
51-8	10 mm	20 mm				
51-9	15 mm	20 mm				
51-10	10 mm	12 mm				
51-11	12 mm	20 mm				
51-12	15 mm	20 mm				
51-13	15 mm	20 mm				
52-1	14 mm					
52-2	17 mm					
52-3	16 mm					

Sample ID	PA0750	MRSA	PA01	ATCC17978	AB030	PA82
52-4	16 mm					
52-5	15 mm					
52-6	12 mm					
52-7	10 mm					
52-8	13 mm					
52-9	10 mm					
52-10	14 mm					
52-11	15 mm					
52-12	17 mm					
52-13	15 mm			10 mm	15 mm	
52-14	19 mm			10 mm	15 mm	
52-15	18 mm			10 mm	14 mm	
53-1	20 mm	20 mm				
53-2	20 mm					
55-1	18 mm					
56-1	17 mm					
57-3	20 mm	20 mm	20 mm			
57-5	20 mm		20 mm			
58-5	20 mm		20 mm			
58-32	20 mm		20 mm			
58-35	20 mm		20 mm			
90-4	16 mm	22 mm		13 mm	25 mm	

Sample ID	PA0750	MRSA	PA01	ATCC17978	AB030	PA82
100-1	14 mm					
100-2	10 mm					
100-3	13 mm					
100-11	18 mm	15 mm		25 mm	33 mm	
100-14	17 mm					
100-15	20 mm					
100-16	17 mm					

3.2 Fungal selection criteria for secondary metabolites characterization

In this study 570 fungal strains were screened for antibacterial activity via agar plug assays. From this set 73 strains yielded positive results and six fungal isolates among the 73 strains were selected for molecular based species identification, and most importantly metabolite characterization. Fungal strains were selected that showed antibacterial activity against both Gram negative and Gram positive bacteria, and fungi that had effects against only Gram negative bacteria, and fungi that demonstrated antibacterial activities only against the sensitive (PA0750) strain. *P. griseofulvum* (40-10) showed evidence of killing all the tested bacteria. Therefore, this particular strain was left out because it has been reported to produce wide range toxins (156,157). The following isolates were chosen for the next series of investigations, 52-15, 57-3, 58-5, 90-4, 100-2 and 100-14.

3.3 Molecular based species identification of selected fungi

Various molecular markers suitable for fungal species/genus identification were used. ITS, β-tubulin, calmodulin, and the translation elongation factor 1-alpha markers were applied for fungal identification. The amplification and sequencing of the ribosomal ITS region is not sufficient for the species identification for many fungal taxa (158). However, the ITS regions can provide sequences suitable for assigning fungi to a Genus. Usually it is very important to use additional molecular markers for species identification. Therefore, in this work, the β-tubulin (*benA*) gene was applied as a secondary marker for species identification among *Penicillium* strains (159). In addition, calmodulin (*caM*) was also used as marker for characterizing *Penicillium* strains (146). The translation elongation factor 1-alpha (*tef1*), was applied for strains that could be related to *Trichoderma,* based on morphological features (147).

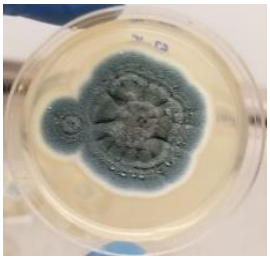
The sequences obtained from various amplified molecular regions were analyzed using the NCBI-BLAST search tool. BLASTn analysis with sequences obtained from the selected strains yielded "hits" with sequences contained within the Genbank database (Table 3.2). Genus affinity for 57-3 and 58-5 isolates were identified, but not species level (57-3 is *Trichoderma* spp. and 58-5 is *Fusarium* spp.). Therefore, more specific search tools were needed to obtain species identity for members of these two genera (160). For species identification, a database called International Subcommision on *Hypocrea* and *Trichoderma* (ISHT) (URL: http://www.isth.info/tools/blast/index) was used for *Trichoderma* spp. identification, and the Westerdijk Fungal Biodiversity Institute database (URL: http://www.wi.knaw.nl/fusarium/**)** was applied for *Fusarium* spp. identification (151,152). With the aid of these databases the analyses of the sequence data of the amplified regions, (*benA*) gene, calmodulin (*caM*), ribosomal ITS, and translation elongation factor 1-alpha (*tef1*) showed that 57-3 sample is *Trichoderma harzianum*, and 58-5 sample is *Fusarium solani* (Table 3.2) (Supplementary material 2).

Table 3.2: Molecular identification of selected fungi. (ITS= Internal transcribed spacer)

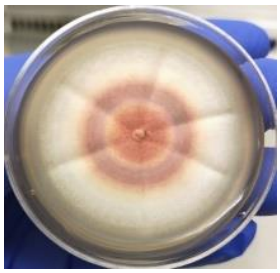
Strain number	Amplified region	Query coverage	Identity percentage	Subject: Accession numbers	Fungus identity
52-15	ITS	100%	100%	AF033476	*Penicillium aurantiogriseum*
	β-tubulin	99%	98.39%	FJ004386	
	Calmodulin	95%	100%	JX141571	
57-3	ITS	91%	100%	KU319055	*Trichoderma harzianum*
	β-tubulin	78%	97.44%	LT707601	
	Calmodulin	74%	100%	FJ442368	
	Translation elongation factor	97%	97.80%	KF923284	
58-5	ITS	100%	99.06%	GQ229075	*Fusarium solani*
	β-tubulin	99%	98.96%	KX240355	
	Translation elongation factor	99%	99.66%	MG857313	
90-4	ITS	100%	99.81%	KX858872	*Penicillium citrinum*
	β-tubulin	100%	99.78%	KC345003	
	Calmodulin	100%	87.26%	AY678555	
100-2	ITS	100%	99.61%	MH856399	*Talaromyces ruber*
	β-tubulin	100%	98.80%	LT559076	
	Calmodulin	93%	99.76%	JX315641	
100-14	ITS	100%	99.64%	JX119035	*Trichoderma atroviride*
	Calmodulin	44%	97.77%	XM_014086200	
	Translation elongation factor	91%	96.76%	KX463439	



Penicillium citrinum



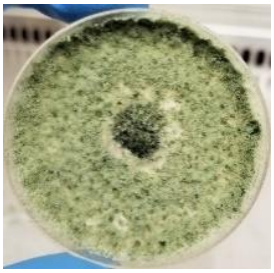
Penicillium aurantiogriseum



Talaromyces ruber



Trichoderma atroviride



Trichoderma harzianum



Fusarium solani

Figure 3.1: Diversity in morphology of the selected strains on solid media (Malt Extract)

3.4 Chemical analysis of the extracted fungal secondary metabolites

3.4.1 Bioactivity of fungal spent media crude extracts

A disc diffusion assay was performed to check the bioactivity of the extracted secondary metabolites. This is to ensure that the intended metabolites have been extracted before proceeding with the chemical analysis. The yield of crude extract is shown in (Table 3.3). 10% of crude weight dissolved in DMSO to determine the antimicrobial activity of each of the extract.

Table 3.3: The weight (mg) of extracted crude from the selected fungal strains

Strains and controls	Crude weight (mg)
52-15	188
57-3	144
58-5	205
90-4	347
100-2	176
100-14 ME media	400
100-14 YESD media	406

3.4.2 High-performance liquid chromatography (HPLC)

HPLC has numerous applications that can be helpful in analyzing chemical compounds. Samples are analyzed via HPLC for three main reasons. First, HPLC can provide an idea of how many compounds does the crude extract from fungal strain contain. Second, this step gives us an indication of the relative concentrations for each of the separated compounds. All fungal samples including the controls (ME media and YESD media) showed a variety of peaks with various retention times (Figs. 3.2-3.10). Every peak represents a fungal product/secondary metabolite.

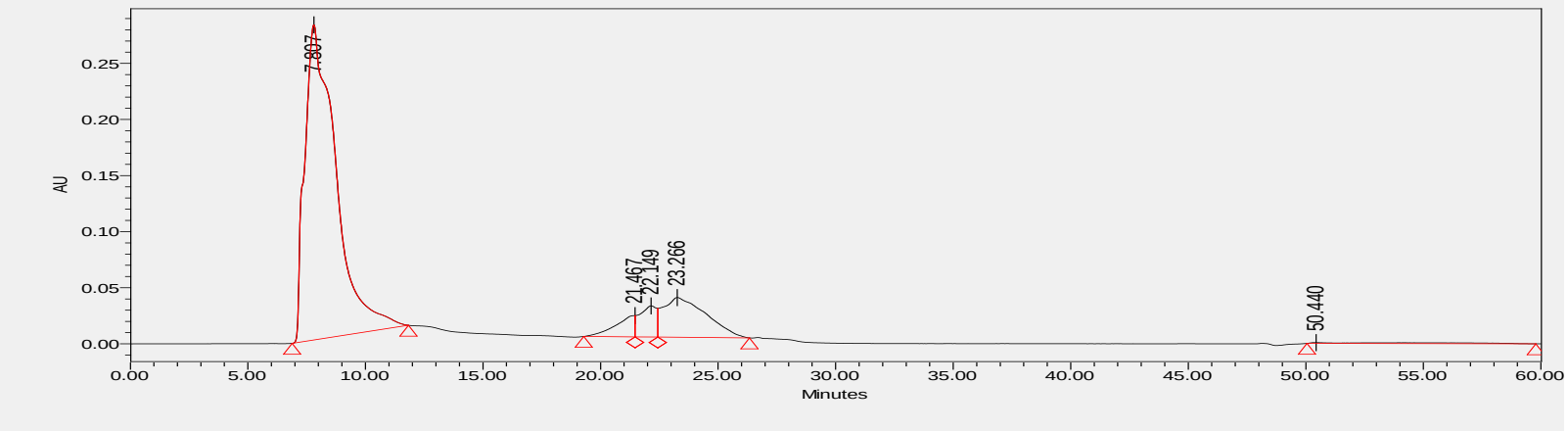


Figure 3.2: HPLC of control ME media. Four peaks were detected with retention times 7.80, 21.46, 22.14 and 23.26 minutes. The peaks that are showing in the figures between 45 and 60 minutes are noise peaks.

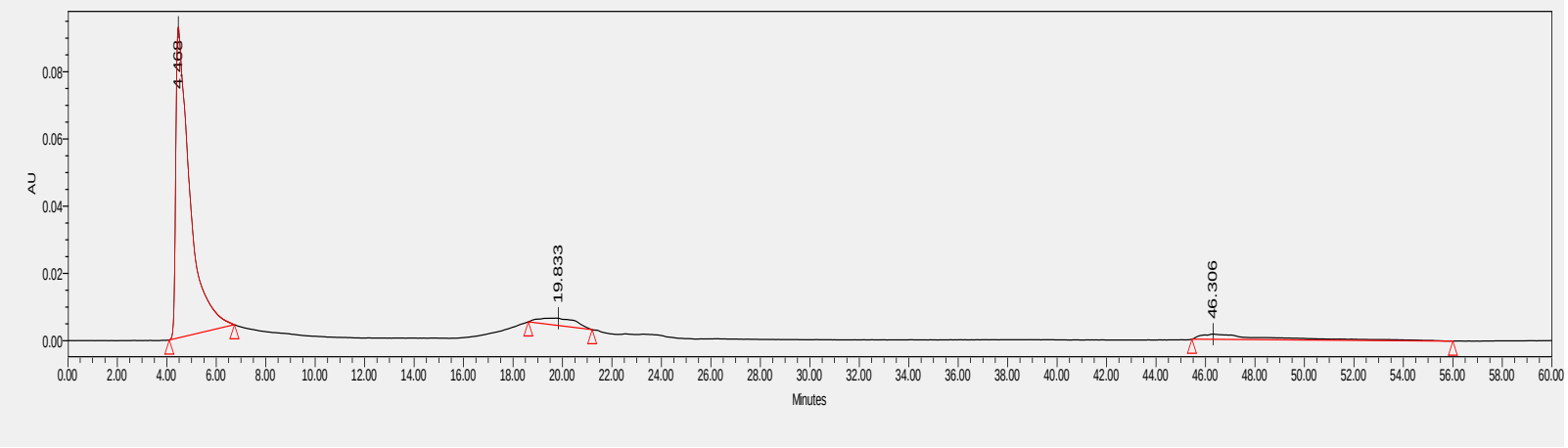


Figure 3.3: HPLC of control YESD media. Two peaks were detected with retention times 4.46 and 19.83 minutes.

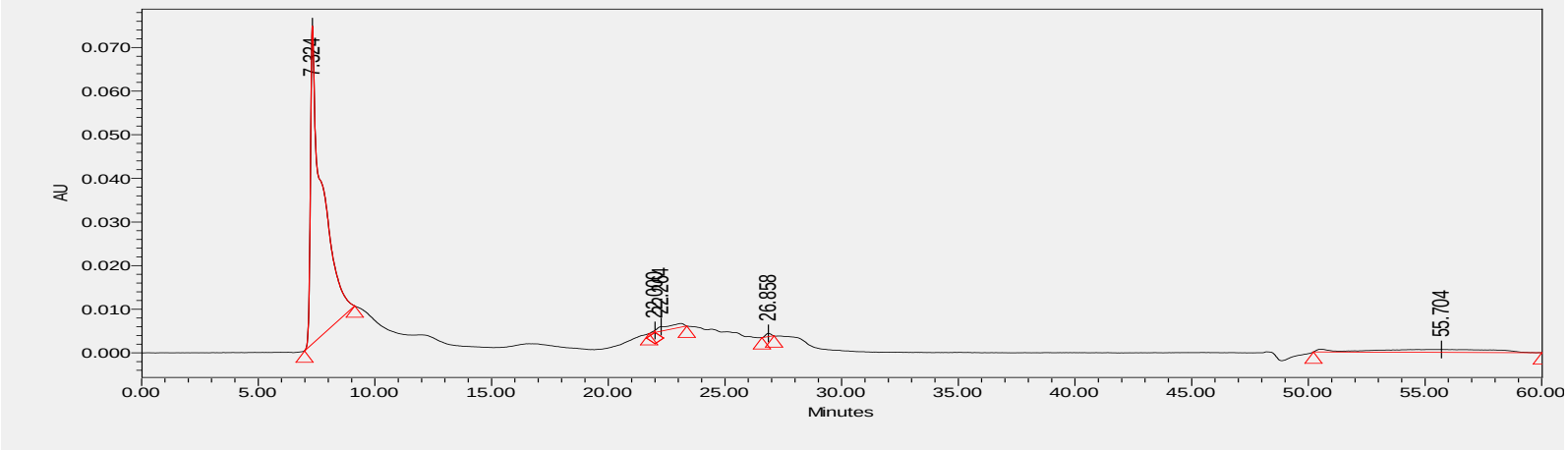


Figure 3.4: HPLC of the *Penicillium aurantiogriseum* (52-15) extract. Four peaks were detected with retention times 7.32, 22, 22.26 and 26.85 minutes.

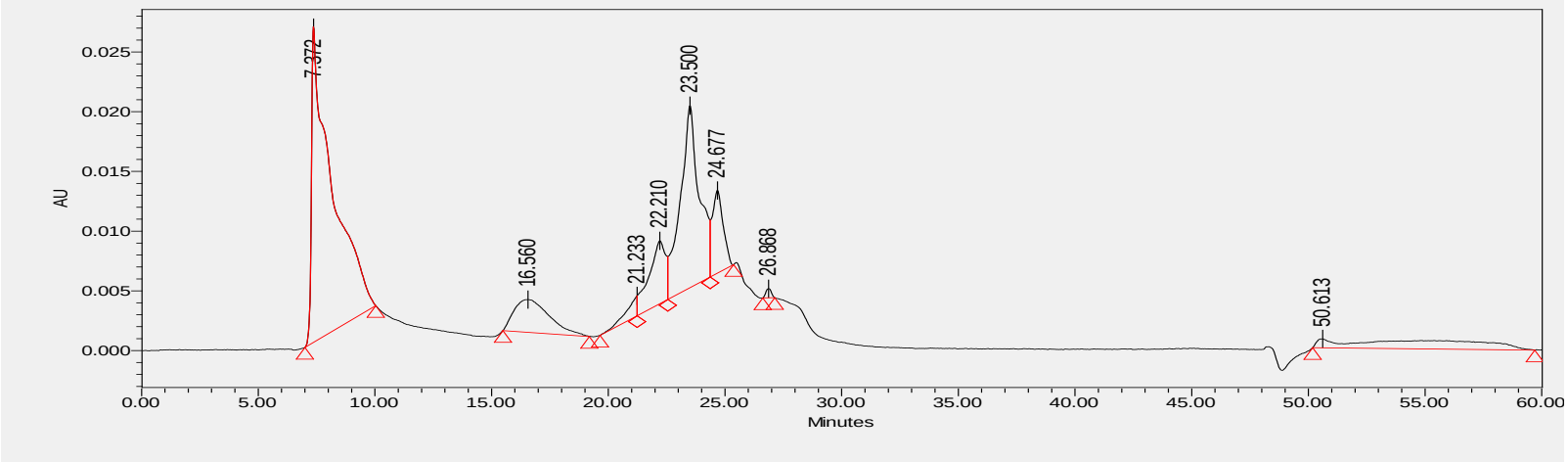


Figure 3.5: HPLC of *Trichoderma harzianum* (57-3) extract. Seven peaks were detected with retention times 7.37, 16.56, 21.23, 22.21, 23.50, 24.67 and 26.85 minutes.

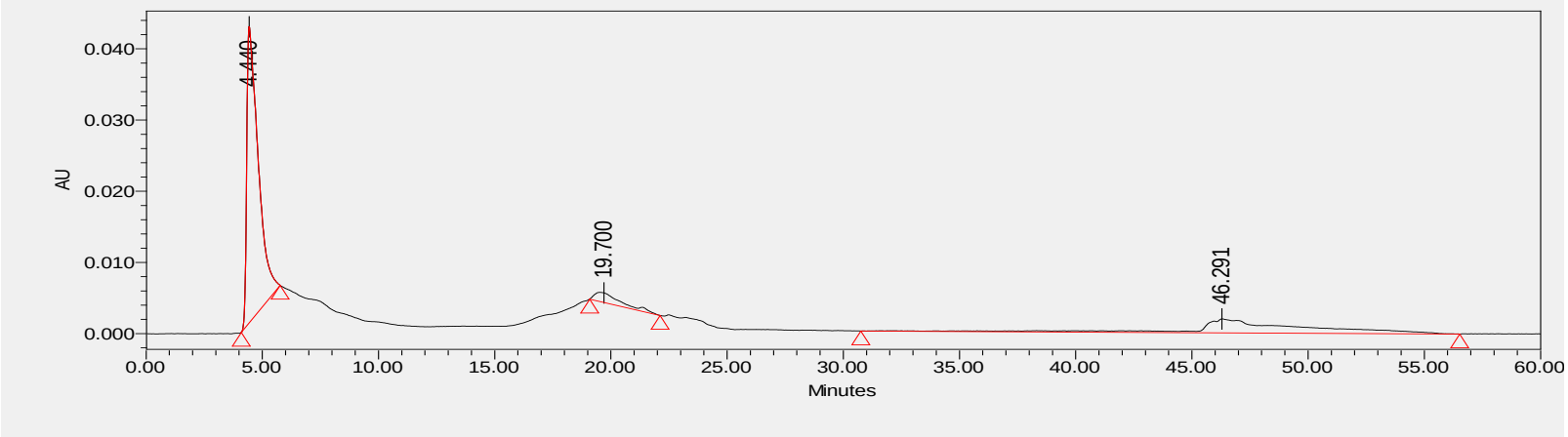


Figure 3.6: HPLC of *Fusarium solani* (58-5) extract. Two peaks were detected with retention times 4.44 and 19.70 minutes.

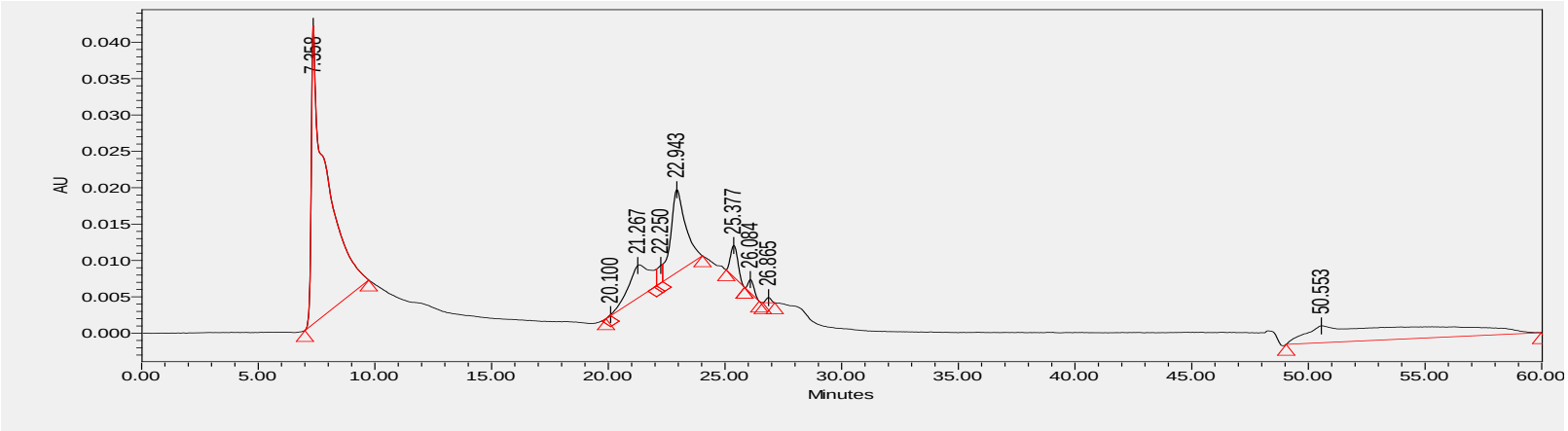


Figure 3.7: HPLC of *Penicillium citrinum* (90-4) extract. Eight peaks were detected with retention times 7.35, 20.10, 21.26, 22.25, 22.94, 25.37, 26.08 and 26.86 minutes.

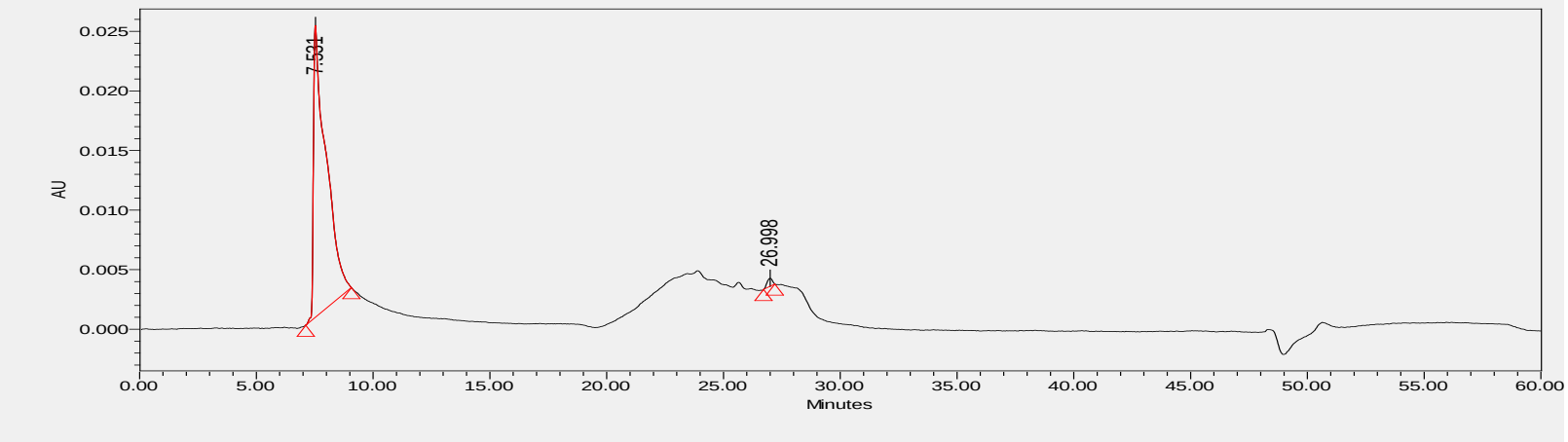


Figure 3.8: HPLC of *Talaromyces ruber* (100-2) extract. Two peaks were detected with retention times 7.53 and 26.99 minutes.

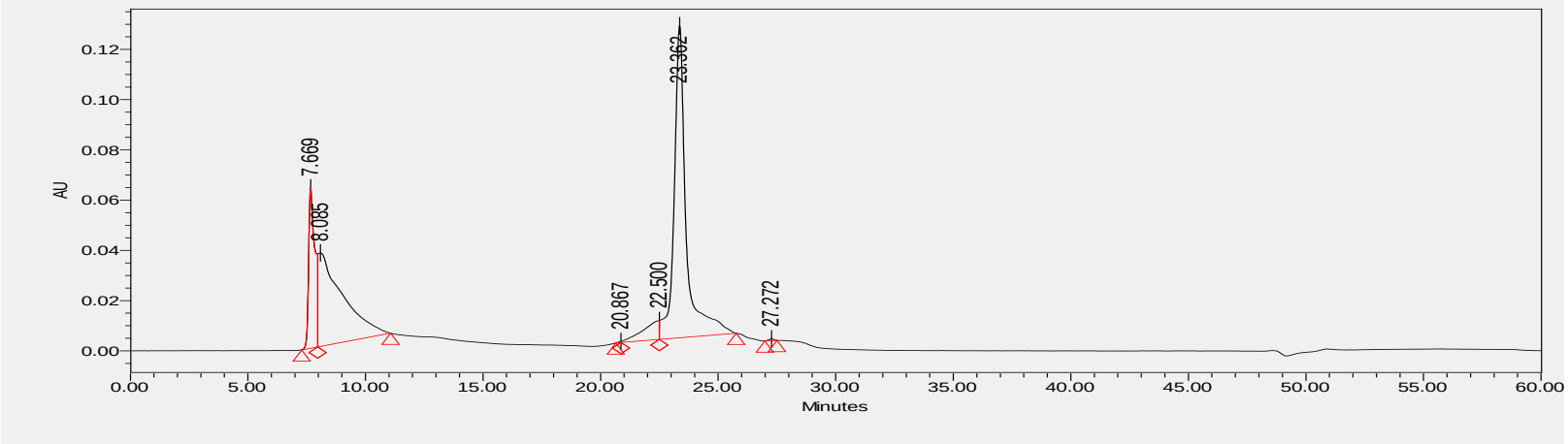


Figure 3.9: HPLC of *Trichoderma atroviride* (100-14 in ME media) extract. Six peaks were detected with retention times 7.66, 8.08, 20.86, 22.50, 23.36 and 27.27 minutes.

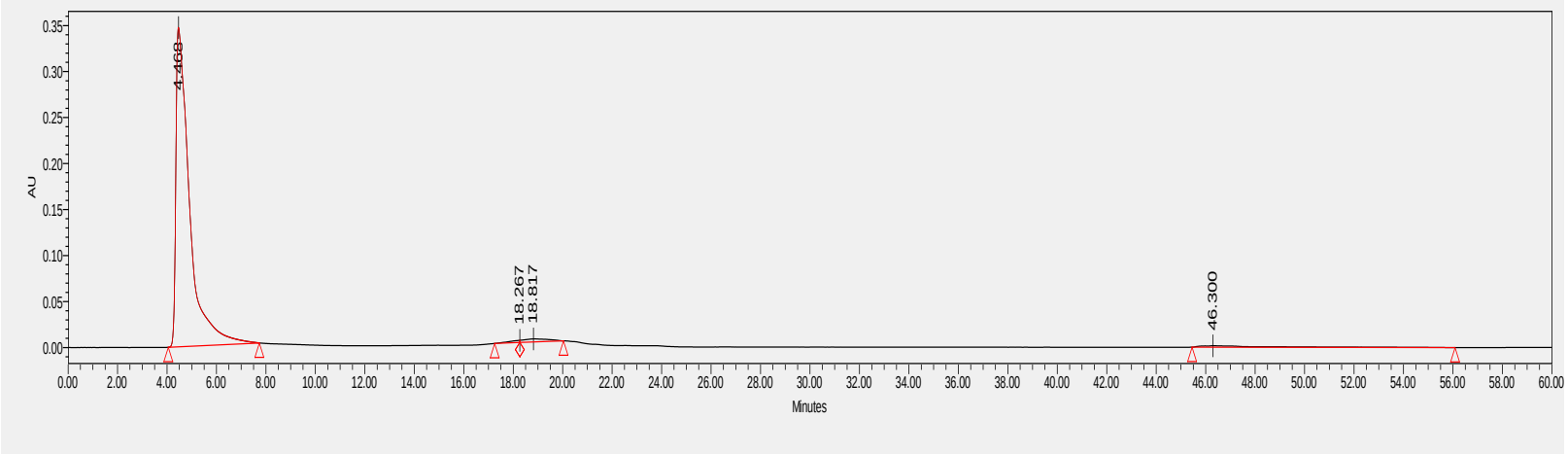


Figure 3.10: HPLC of *Trichoderma atroviride* (100-14 in YESD media) extract. Three peaks were detected with retention times 4.46, 18.26 and 18.81 minutes.

3.5.2 Liquid chromatography–mass spectrometry (LC-MS) preliminary analysis

LC-MS data can identify the fungal strains that could be a good source of new secondary metabolites. Preliminary **LC-MS** results of fungal extracts in this study, showed that some secondary metabolites appeared to be produced with different biological activities, but these have been previously discovered and studied. However, some of the LC-MS results suggested that there are some fungal products in the samples that could be unique. Therefore, based on this preliminary data based on LC-MS analysis, fungal strains that showed promising results can be further investigated to get more information about their secondary metabolites.

The LC-MS analysis as shown in the heat map (Fig. 3.11), provided an overview of compounds present in crude fungal extracts and some of these compounds generated signals that are not present in currently available databases (labeled as “no hit”), which means that our fungal strains might be producing new compounds that have not yet been identified. It is worth to note that the biological activities of previously discovered fungal products that were produced by my fungal strains showed that some have anticancer, anti-inflammatory, antifungal, antiviral, antibacterial, and other biological effects (supplementary material 1).

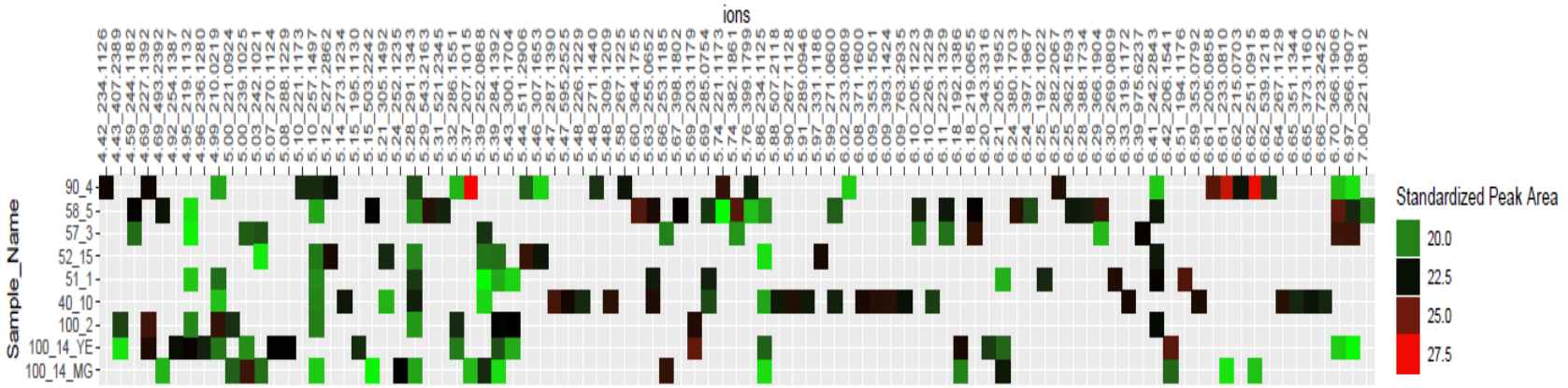


Figure 3.11: Heat map of fungal extracts showing retention time and ion mass *m/z*. Green color represents the lowest abundance level, while red color represents the highest abundance level of SMs.

Chapter 4: Discussion

4.1 Isolation of fungi and phenotypes based on agar plug assays

Soils contain a diverse set of microorganisms that interact and compete for nutrients to survive (161). Microbial interactions can range from mutualism to parasitism and there can be competition for resources. These interactions between microorganisms can be considered a form of stress that triggers the release of chemical signals (i.e. secondary metabolites) (162-164). For this study, soil samples were collected from different shore lakes in Manitoba to isolate fungi. Also, *Drosophila* sp. (fruit fly) was chosen as a source for fungi. Most of the fungi isolated from soil samples were either *Penicillium* or *Aspergillus species*. The SMs of these two fungi have been studied a lot. Therefore, this study focused on the chemistry of other genera besides *Penicillium* or *Aspergillus*, as this could provide a better chance to find new SMs. The reality is that in many studies one tends to "re-discover" previously characterized SMs; the goal is to discover new SMs. The total number of fungi isolated during this study was 570 and only 73 fungal isolates were able to inhibit bacterial growth, based on agar plug assays, of the efflux deficient *P. aeruginosa* strain PA0750. Suggesting these 73 fungal strains are producing SMs with antibacterial activities.

These 73 fungal strains were challenged with various Gram negative bacteria such as the wild types for *P. aeruginosa* and *A. baumannii,* and clinical isolates and multi-drug resistant strains of *P. aeruginosa* and *A. baumannii,* and the Gram positive methicillin-resistant *Staphylococcus aureus*. The antibacterial effects or profiles of these fungi against the various

bacterial strains were different (Table 2.2). Challenging these fungi against these bacteria provides a better idea of the nature of the antibacterial properties of the SMs. Some fungi appear to produce compounds only effective against the efflux deficient strains other fungi produce compounds that target clinical strains and MRSAs. All of these SMs might be useful in future strategies of developing antibiotics or treatments against bacterial infections. For example, compounds showing only activity against efflux deficient strains could be combined into cocktails that could be effective in clinical settings. Developing antibiotic resistance against a cocktail of drugs would be relatively slower compared to antibiotic resistance evolving against one strong "drug". In the long term this study recovered fungal isolates and SMs that deserve to be studied in more detail with regards to their antibacterial activities.

4.2 Molecular Taxonomy

Fungi were selected for more detailed analysis based on their ability to produce SMs possessing various antibacterial effects. There was one fungal isolate (40-10) that was able to inhibit the growth of all tested bacterial strains. Molecular identification of that isolate revealed that this isolate is *P. griseofulvum.* This species has been well characterized and is known to be a source of many toxic secondary metabolites (156,157). Therefore, this species was not further analyzed in this study as the presence of toxins would make this fungus challenging for isolating new SMs that could have antimicrobial and be non-toxic to mammals.

The molecular identification of some fungal isolates was quite challenging. The two isolates 57-3 and 58-5 after ITS region amplification and sequencing were identified to genus level as *Trichoderma* and *Fusarium*, respectively. For potential species level identification, the

three additional markers were amplified: portion of the β-tubulin (*BenA*), calmodulin (*CaM*) gene, and translation elongation factor 1-alpha (*tef1*) genes for the 57-3 and 58-5 isolates. In addition to that, more specific databases for *Trichoderma* and *Fusarium* were applied for species identification. After analyzing these sequences, the results showed that isolate 57-3 is *Trichoderma harzianum*, and 58-5 isolate is *Fusarium solani*. Identification of *Trichoderma* and *Fusarium* requires more molecular markers in order to resolve species level identification. Species concept within the genera *Trichoderma* and *Fusarium* are very challenging, historically mycologists have applied a combination of characters such as molecular data (165), physiological and morphological data and when applicable host species and geographic origins.

4.3 Preliminary chemical analysis of secondary metabolites present in crude extracts

HPLC analysis is very helpful step for SMs purification and identification. Data obtained from HPLC analysis provides us information such as the potential number of SMs produced by the fungus, the relative abundance of each SM in the crude extract, and provides retention times for the various SMs which can be used when applying preparative HPLC strategies to collect fractions enriched in certain SMs. HPLC results for 58-5 and 100-14 (in YESD) as shown in Figs. 6 and 10, showed lower number of peaks and very low concentration of SMs present in their crude extracts compared to HPLC results of the other crude samples in this study. The HPLC analysis of extracts obtained from 58-5 and 100-14 in (YESD) was done five months after the crude extraction. Here the possibility exists that some SMs might have decomposed due to the long storage time. While, HPLC analysis for the other crude extracts was done immediately after the crude extracts had been prepared.

The concentrations, based on the size of the peaks, of these secondary metabolites were mostly low; therefore, growth conditions need to be altered to induce the production of fungal secondary products. It has been reported that "stress" can induce the production of secondary metabolites in fungi (166-169). This would hopefully have facilitated the generation of fractions suitable for further chemical analysis and identification.

The LC-MS analysis of fungal crude extracts revealed interesting information about SMs present in the various fungi being examined (Table 4.1). A variety of different SMs were produced by the studied fungal isolates as shown in the heat map (Fig. 3.11). The heat map showed that some SMs were unique to a specific fungus, and several SMs were shared between different fungi. For example, the isolate 58-5, produced 6 unknown SMs. Also, isolate 58-5 and 100-14 (in ME) produced the same unknown SM, with retention time at 4.69 minutes, and the ion mas is (*m/z* 493.2392). Preliminary analysis of *P. griseofulvum* (40-10) extracts showed that this fungus produced high numbers of highly toxic SMs (supplementary material 1). Obviously one needs to apply more analytical chemistry techniques to identify and study these unknown SMs, these may be novel compounds with economic relevance.

Table 4.1: Showing some of previously discovered SMs that were detected in crude extracts of our fungal collection

Compound	Biological activity	Reference
Inotilone	Anti-inflammatory activities	170,171
Harzianic acid	Antifungal and plant growth promoting activity	172
Pulcherriminic acid	Antifungal and antibacterial activities	173,174
Tomaymycin	Anticancer properties	175,176
Maculalactone E	Antibacterial effect	177,178
Salinosporamide K	Anticancer properties	179,180

For *Fusarium solani* (58-5), LC-MS detected 29 signals for different secondary metabolites (SMs). Six of those are not in the currently available database, and therefore labeled as “no hit”. The other twenty SMs were natural products previously noted in plants, fungi, and bacteria. Based on previous studies on endophytic fungi, some endophytic organisms have the ability to produce biochemical compounds similar or identical to those produced by their host plant (181). For example, an *Eupenicillium* spp. endophytic on *Murraya paniculata* produces alanditrypinone, one of the rare classes of spiroquinazoline alkaloids (182, 183). The biological activity of the product is still unknown (181). Acacetin, is potentially a plant derived compound was detected in *Fusarium solani* (58-5) crude extract. This compound has gained scientists attention due to its numerous pharmacological properties, including neuroprotective, cardioprotective, anticancer, anti-inflammatory, anti-diabetic, and antimicrobial activities (184).

Stachyline C, is a SM previously reported from the marine-derived fungus *Stachylidium* spp. (185). This compound was detected in the crude extract of 58-5. This SM also possess a wide range of pharmacological properties, including tumor cell cytotoxic activity, antidiabetic activity, protein kinase inhibition, NF-κB protein complex inhibition, antibacterial and antifungal activity, antiplasmodial activity, and antiviral activity (HIV and influenza viruses) (185).

One bacterial SM previously noted in a *Micrococcus* sp., known as limazepines B1, was detected in the *Fusarium solani* (58-5) fungal extract. This example shows that different microorganisms could share the production of similar or related SMs. This SM was shown in a previous study to have a broad antibacterial spectrum (186). LC-MS analysis has detected a large number of SMs in the rest of the fungal samples (supplementary material 1). A heat map

was generated for the SMs produced by the various fungal strains. The heat map shows the ion mass detected by LC-MS analysis for the various compounds and the abundance of each compound and which fungus is the source of these products (Fig. 3.11).

In this study, the effect of the production of SMs using two different media was also investigated. In a previous study, it was found that fungal strains yielded large numbers of SMs when grown in YESD media or ME media compared to other media (187). Therefore, in this study for the fungal strain *Trichoderma atroviride* (100-14) YESD was used along with ME media for the comparison. LC-MS showed that the number of the SMs produced by strain 100-14 grown in YESD media, was 21 SMs, and one compound labeled as no hit. While, strain 100-14 crude extract from ME media, produced 18 SMs, with two SMs labeled as no hit. Interestingly, not only the number of the SMs produced vary, the types of compounds identified also varied. For example, only six SMs were found in both media, while the rest of SMs were only found either in ME or YESD media although the source was from the same fungus (Table 4.2 and Fig. 3.11). This suggests that the fungal medium can influence the range of SMs produced by the fungus.

Table 4.2: Comparison of SMs of *Trichoderma atroviride* (100-14) grown in YESD and ME media

SMs detected in both media	SMs detected only in ME	SMs detected only in YESD
g-L-Glutaminyl-4-hydroxy-benzene	trans-Zeatin	Harzianic acid
epsilon-Cadinene	Pulcherriminic acid	Nb-Acetyltryptamine
N-Phenethyl-valeramide	Maculalactone E	Lycorine
N-Phenethylbutyramide	Limazepine B1	Monascuskaochroman
10-Hydroxy-trans-paspalic acid	Rigidiusculamide A	cyclo[L-(4-Hydroxyprolinyl)-D-leucine]
2-(1H-Indol-3-yl)ethyl 2-hydroxypropanoate	Citrinolactone B	cyclo(L-Pro-L-Pro)
	5-Hydroxytryptophan	cyclo-(D-Leu-L-Trp)
	Salinosporamide K	Polanrazine A

Chapter 5: Conclusion and future work

Fungi are a promising source of many secondary metabolites. They appear to be a great source of antibacterial agents. Thus, in order to fight bacterial resistance, a great effort should be applied in mining for new antimicrobial agents and adjuvants to restore the currently available antibiotics. The main objective of this study is to find novel and safe fungal secondary metabolite that could be used as antibiotics or adjuvants of antibiotic that are able to cure infections with opportunistic pathogens of *P. aeruginosa* or, *A. baumannii*. The initial survey based on screening 570 fungal strains showed that fungi with antimicrobial activities can be identified by agar plug assays with the help of a *P. aeruginosa* strain that lacks efflux pumps. This approach yielded 73 fungal strains of which six were explored in more detail. In the future, more fungi should be isolated from under-sampled niches, such as marine environments, epiphytes, extreme environments, fungi that exist in symbiotic relationships with insects etc. This study also showed that media can affect the production of secondary metabolites in some fungi. It would be highly desirable to examine SM production for some of the studied fungi by growing them in various different media and under different conditions (pH, temperature etc.).

For some fungi there is the promise of discovering novel compounds. For example, based on LC-MS analysis of the fungal strain *Fusarium solani* (58-5) crude extract, we found that this strain produced nine SMs that are not in the currently database suggesting that they may be novel. Therefore, further investigation using various analytical chemistry techniques such as preparative HPLC, Nuclear Magnetic Resonance (NMR) spectroscopy, and LC-MS might help us to identify these SMs, and bioactivity assay to determine their antibacterial effect.

Although fungi can be a source of new antibiotics or drugs, they are also known to produce many types of mycotoxins. Therefore, the compounds recovered from fungi have to be assessed with regards to their toxicity towards eukaryotic cells. The toxicity of SMs can be measured by means of various assays. Such as by using animal blood cells and examine the effect of SM on the stability these cells. If the SM has a hemolytic effect it could be an indication that the SM promotes cell lysis and could therefore be toxic to eukaryote cells. This type of toxicity method is convenient, and fast (188). In addition, the hemolytic effect can be measured as high, low, or absent (189). However, more detailed toxicity assays would have to be performed in order to assess the level of safety of fungal SMs, some SMs may not affect membranes but could target specific pathways that can result in cell death.

The long term goal is to find biologically active SMs and produce them in sufficient amounts to allow for more detailed chemical analysis and potentially clinical assays. This would require the optimization of fungal growth conditions and SMs extraction protocols. It would also be very beneficial to identify the gene clusters or key genes associated with production of SMs of interest. Genomics offers an opportunity to examine fungal genomes for the presence of biosynthetic gene clusters associated with the production of SMs. Bioinformatics tools are available to annotate fungal genomes and identify gene(s) associated with the production of SMs. In addition, it appears that many SM genes are cryptic and thus their expression is still unknown. These “cryptic” genes offer an untapped resource for the production of secondary metabolites. It would therefore be ideal if both the metabolome and the genomes along with transcriptomes would be characterized for fungi that showed promise during this study. These data might identify genes that could be isolated and moved into other fungi that are more suitable for genetic manipulation and growth in the large scale fermenters. Heterologous expression of genes in

Aspergillus spp., or baker's yeast *Saccharomyces cerevisiae* might allow for expressing genes from various fungal sources. This would allow for optimizing the expression of these genes and producing higher amounts of the desired SMs (190). This would address the problem of low and sometimes inconsistent SM production by the original fungal species where these compounds were noted. It also offers a strategy to examine the activity of genes that are part of biosynthetic gene clusters by show no expression in the context of their original genomes. Manipulating their promoters and moving these genes into a fungus such as *Aspergillus* would offer the opportunity to find new SMs (191).

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Supplementary Table 1

Sample	ions	row.m	row.re	Peak	Log 2	ranne	Closes	nnn	Numb	X1	X1.1	X2	X2.1	X3	X3.1	X4	X4.1	X5	X5.1	X6	X6.1	X7	X7.1	X8	X8.1	X9	X9.1	X10	X10.1	X11	X11.1	X12	X12.1	X13	X13.1	X14	X14.1	X15	X15.1	X16	X16.1	X17	X17.1	X18	X18.1	X19	X19.1	X20	X20.1	X21	X21.1	X22	X22.1	X23	X23.1	X24	X24.1	X25	X25.1	X26	X26.1	X27	X27.1	X28
51_1	6.41_242.284	242.3	6.41	7E+06	22.78	242.28 18771	NA	NA	NA																																																												
51_1	5.39_252.086	252.1	5.39	2E+05	17.75	252.08 42791	NA	NA	NA																																																												
51_1	6.51_194.117	194.1	6.51	2E+07	24.31	194.11 56588 24-	194.1	0.186	4	p-Hydroxyphe	[B]	Fusaric acid methyl		MDMA; Methyl	[synth etic]	4-(Acetylamino																																																																	
51_1	6.30_269.0809	269.1	6.3	1E+07	23.19	269.07 82091 91- 269.08 35908 09	269.1	0.208	18	4'-Hydroxy-5-methoxyflavone	[F] Beauveria bassiana + 5-methoxyflavone	Formoneti n; Biochanin B; Daidze in 4'-methyl ether;	[F] marine fungus 09540 7	7-Hydroxy-8-methoxyflavone	[F] Aspergillus flavus + 7,8-dimeth oxyflavonero duced	6-Methyluracil	Hallachrome		Vesparione	[S] Metarhizium vesparium	Digitolutein		Phomarin-6-methyl ether		Rubiadin-1-methyl ether		Zigane	1-Hydroxy-6-methoxy-8-methylanthraquinone	[B] Streptomyces sp. GW 24/1694	10-Hydroxy-5-methoxy-2-methyl-1,4-anthraquinon	[synth etic]	Chrysophanol 8-methyl ether	[B] marine derived Nocardia sp. ALAA 2000	Xanthopurpurin-dimethyl ether		Isochrysophanol 8-methyl ether		Rubiadin 3-methyl ether		Madeirin		1-Hydroxy-5-methoxy-2-methylanthraquinone																		

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Supplementary material 2

List of sequences

Fungus identity: *Penicillium aurantiogriseum*

Amplified region: ITS sequence

Top hit: Accession number: AF033476

>52-15 F. (650 bp) _merged_52-15 R. (650 bp)

ATTGATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACGACCCCCCA
GAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGG
TTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTTTGGGTCCAA
CCTCCCACCCGTGTTTATTTTACCTTGTTGCTTCGGCGGGCCCGCCTTTACTGGCCGC
CGGGGGGCTTCACGCCCCCGGGCCCGCGCCCGCCGAAGACACCCTCGAACTCTGTCT
GAAGATTGAAGTCTGAGTGAAAATATAAATTATTTAAAACTTTCAACAACGGATCTC
TTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCA
AATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGG
GCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCG
TCCTCCGATTCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCT
CGAGCGTATGGGGCTTTGTCACCCGCTCCGTAGGCCCGGCCGGCGCTTGCCGATCAA
CCCAAATTTTTATCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAG
CATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCCAGTAACGGCGAGT
GAAGCGGCAAGAGCTCAAATTTGAAAGCTGGCTCCTTCGGGGTCCGCATTGTAATTT
GCAGAGGATGCTTCGGGAGCGGTCCCCATCTAAGTGCCCTGGAACGGGACGTCATA
GAGGGTGAGAATCCCGTATGGGATGGGG

Fungus identity: *Penicillium aurantiogriseum*

Amplified region: β-tubulin sequence

Top hit: Accession number: FJ004386

>52-15 F. (434 bp) _merged_52-15 R. (649 bp)

AACATCTGATCAGATGATGCACGATTATTCGGTTTCCAGTCGTTGCACTCACATGGT
TGAAGTAGACGTTCATACGCTCGAGCTGAGTCGGGACCCCTTTTCCCTACCAGATAT
CCGAGGTGTGATCTACCGGAAACCCCCATCACCATTAGAACTTACTGTCCATCGCCA
TCGAGACCGTGCTCACCAGAGATGGTTTGCCCCTAATCGAGTTGGTAACCCAATGGT
GCTGCTTTCTGGTAAGTGCCGAGCTTTTTTTCCCCTTCGCGTTGGGTACAATTGACAG
GTTACTAACTCGATTACAGGCAAACCATCTCTGGCGAGCACGGTCTCGATGGCGATG
GACAGTAAGTTCTAATGGTGATGGGGGTTTCCGGTAGATCACACCTCTGATATCTTG
CTAGGTACAATGGTACCTCCGACCTCCAGCTCGAGCGTATGAACGTCTACTTCAACC
ATGTGAGTGCAACGACTGGAAACCGAATAATCGTGCATCATCTGATCAGATGTTTTT
CTTTGATAATCTAGGCCAGCGGTGACAAGTACGTTCCCCGTGCCGTTCTCGTCGATTT
GGAGCCCGGTACCATGGACGCTGTCCGCTCCGGTCCTTTCGGCAAGCTTTTCCGCCC
CGACAACTTCGTCTTCGGTCAGTCCGGTGCTGGTAACAACTGGGCCAAGGGTCACAA
AATGAGGGTA

Fungus identity: *Penicillium aurantiogriseum*

Amplified region: Calmodulin sequence

Top hit: Accession number: JX141571

>52-15 F. (595 bp) _merged_52-15 R. (476 bp)

GCCTTCTCCCTGTTCGTGAGTGACTCTACATAAAAATCGAAGACGTGAAGGTCGAAT
ATGCTGACCGAGAGTTTGTTTTGTTGCGGAATAGGACAAGGATGGTGATGGTACGTG
TGGTCGCGCCCGACTGTTCAGTTGACAGCAGTGTCCTCTGTGATCGAATTTCAAGAG
AAAAGTATCCTAACATACAATTCTCCCAATAGGACAAATCACCACTAAGGAGCTGG
GCACCGTCATGCGCTCGCTCGGTCAGAACCCCTCCGAGTCCGAATTGCAGGACATGA
TCAACGAGGTCGACGCCGACAACAACGGAACCATTGACTTCCCCGGTACTTGACCA
TAACCCACTGGTATAATCGAGAGACGGCTATTGACGTGCGATAGAATTCCTCACCAT
GATGGCTCGTAAAATGAAGGACACTGATTCCGAGGAGGAGATCCGTGAGGCTTTCA
AGGTCTTCGACCGCGACAACAACGGCTTCATTTCCGCCGCTGAGCTCCGCCACGTCA
TACCTTCCATCGGAGAATCAGTGTCCTTCATTTTACGAGCCATCATGGTGAGGAATT
CTATCGCACGTCATAGCCGTCTCTCGATTATACCAGTGGGTTATGGTCAAGTACGGG
GAAGTCAATGGTT

Fungus identity: *Trichoderma harzianum*

Amplified region: ITS sequence

Top hit: Accession number: KU319055

>57-3 F. (558 bp) _merged_57-3 R. (605 bp)

CGTACCCATCACGTTGTTGTGGTTAACTCCCGAACCTCCTCCGTAGGGTGACCTGCG
GAGGGATCATTACCGAGTTTACAACTCCCAAACCCAATGTGAACGTTACCAAACTGT
TGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCGCAGCCCCGGACCAAGGCGCCCGC
CGGAGGACCAACCAAAACTCTTATTGTATACCCCCTCGCGGGTTTTTTTATAATCTG
AGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTTCAACAACG
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATT
CTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGTCG
GCGTTGGGGATCGGCCCTCCCTTAGCGGGGTGGCCGTCTCCGAAATACAGTGGCGGT
CTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGGGAGCGCGGCGCG
TCCACAGCCGTTAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATA
CCCGCTGAACTTAAGCATATC

Fungus identity: *Trichoderma harzianum*

Amplified region: β-tubulin sequence

Top hit: Accession number: LT707601

>57-3 F. (398 bp)

GGTCTCGACAGCAATGGTATCTATGGAGGCTCTTCCGAGCTCCAGCTCGAGCGCATG
AACGTCTACTTCAACGAGGTACGTGTTTCCTTTTGCCAGCTAGACGCGCCACTCGAG
AAGCCCCTATTCTGACCTCGGATACTGCAGGCCAACAACAACAAGTATGTTCCTCGC
GCTGTCCTCGTCGATCTTGAGCCCGGTACCATGGACGCCGTCCGTGCCGGTCCTTTC
GGTCAGCTCTTCCGACCCGACAACTTCATCTTCGGCCAATCCTCTGCCGGAAACAAC
TGGGCCAAGGGTCACTACAACTGAGGGTAGGCCAGCTGTTCCGCCCCGACAACTTC
GTTTTCGGCCAGTCTGGTGCTGGTAACAATTGGGCCAAGGGTCACTACACTGAGGGT

>57-3 R. (384 bp)

GCCGAGAGTTGTCGGGTCGGAAGAGCTGACCGAAAGGACCGGCACGGACGGCGTCC
ATGGTACCGGGCTCAAGATCGACGAGGACAGCGCGAGGAACATACTTGTTGTTGTT
GGCCTGCAGTATCCGAGGTCAGAATAGGGGCTTCTCGAGTGGCGCGTCTAGCTGGC
AAAAGGAAACACGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGGAGCTCG
GAAGAGCCTCCATAGATACCATTGCTGTCGAGACCGTGCTCGCCAGAGATGGTCTGC
CAGAAAGCAGCACCGATTGGTTACCATCGCAGGCGTCGCTTCGAGATTTCCGTCACC
GATCTCTAGAGGAAACATACCAGAAAGCAGCACCGATTTGGTTACA

Fungus identity: *Trichoderma harzianum*

Amplified region: Calmodulin sequence

Top hit: Accession number: FJ442368

>57-3 F. (602 bp) _merged_57-3 R. (605 bp)

TCCGAGTACCAAGAGCCTTCTCCCTCTTTGTGAGTCTTTACCTCGTTTCTGGTGGCAA
TTCGGACGGTTTGAGTGCTAACCCAGCTTGCTACAGGACAAGGACGGTGATGGTAC
GTAGCAGCGAGTGACGCGACACCACCCCTCTCCCCCTTTTGCGAACAGGCACCGGA
GCACCGAATGACTTGCTGCCGACCGATATTTCCCTTGCTAGCCCCTATTCACGATAT
CTTAGCAAAAGAGAAACTGACAGGCTTGACCCCGCAGGCCAGATCACCACCAAGGA
GCTGGGCACCGTGATGCGCTCTCTCGGCCAGAACCCCTCCGAGTCAGAGCTTCAGGA
CATGATCAACGAGGTCGATGCCGACAACAACGGATCTATCGATTTCCCCGGTATGTC
AAAAGTCAAAGTATTGGGCCGTTGCCACATTCGAGCTAATACGGAGCGGTGTAGAA
TTCCTGACCATGATGGCACGAAAGATGAAGGACACTGATTCCGAGGAGGAGATCCG
AGAGGCATTCAAGGTTTGTTTCCGGCACTATTATCTATTTTTAGTTTACTATGTTTGC
TAATGTATGCGCTCAGGTCTTTGACCGCGACAACAACGKCTTCATCTCAGCCGCTGA
GCTGCGCCATCATGACCCCATTCGG

Fungus identity: *Trichoderma harzianum*

Amplified region: Translation elongation factor sequence

Top hit: Accession number: KF923284

>57-3 F. (787 bp) _merged_57-3 R. (783 bp)

CGTACCATCGAGAAGTTCGAGAAGGTAAGCTTCNANTCNTTTTCACCTCGATTCTCC
CTCCACATTCAATTGTGTCCGACAATTYTGCARAGAATTTTCGTGTCAACAATGTTTC
ATCACCCCGCTTTGCATTACCCCTCCTTTGGAGCGACGCAAAATTTTTTTGCTGTCGT
TTGGTTTTAGTGGGTTTTTTTGTGCACCCCACTAGCCTCGTTTTTTYTGCTTCGCTCTC
ACTTCCCAGCCATCATTCAACGTGCTCTGTGTCATCACTTTCAGCGATGCTAACCACT
TTTCCATCAATAGGAAGCCGCCRAACTCGGCAAGGGTTCCTTCAAGTACGCTTGGGT
TCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACCATCGACATTGCTCTGTG
GAAGTTCGAGACTCCCAAGTACTATGTCACCGTCATTGGTATGTTCTTGCCATCAAC
TTGACACAGCGATTACAAGCCAGTGCTAACAAGCAATTCACAGACGCTCCCGGCCA
CCGTGATTTCATCAAGAACATGATCACTGGTACTTCCCAGGCCGATNGCGCTATCCT
CATCATTGCCGCCGGTACTGGTGAGTTCGAGGCTGGTATCTCCAAGGATGGCCAGAC
CCGTGAGCACGCTCTGCTCGCCTACACCCTGGGKGTCAAGCAGCTCATTGTTGCCAT
CAACAAGATGGACAMTGCCAACTGGGCCGAGGCTCGTTACCAGGAAATCATCAAGG
AGACTTCCAACTTCATCAAGAAGGTCGGCTTCAACCCCAAGGCTGTTGCTTTCGTCC
CCATCTCCGGCTTCACG

Fungus identity: *Fusarium solani*

Amplified region: ITS sequence

Top hit: Accession number: GQ229075

>58-5 F. (886 bp) _merged_58-5 R. (849 bp)

GTTGATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACCACCC
AGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAG
GTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTATTCAACTCATCAACCC
TGTGAACATACCTAAACGTTGCTTCGGCGGGAACAGACGGCCCCGTGAAACGGGCC
GCCCCCGCCAGAGGACCCCTAACTCTGTTTCTATAATGTTTCTTYTGAGTAAAACAA
GCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAAC
GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTT
GAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTAC
AACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAGCCCCCCGTGGGCACA
CGCCGTCCCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATCGCGTAGTAGCTAA
CACCTCGCGACTGGAGAGCGGCGCGGCCACGCCGTAAAACACCCAACTCTTCTGAA
GTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGA
GGAAAAGAAACCAACAGGGATTGCCCCAGTAACGGCGAGTGAAGCGGCAACAGCT
CAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGTAATTTGTAGAGGATGCTTTTGGT
GAGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTGAGAGCCYCGTCT
GGTTGGACGCCGAACCTCTGTAAAGCTCCTTCGACGAGTC

Fungus identity: *Fusarium solani*

Amplified region: β-tubulin sequence

Top hit: Accession number: KX240355

>58-5 F. (289 bp) _merged_58-5 R. (295 bp)

TCCATTGGTAACCAAATCGGTGCTGCTTTCTGGCAGACCATCTCTGGCGAGCATGGC
CTCGACAGCAATGGTGTTTACAACGGCACCTCTGAGCTCCAGCTCGAGCGCATGAGC
GTCTACTTCAACGAGGTATGTTGTCCGGCAGCTGTGCGAAATCTAGCTGACATTTGT
AGGCCTCTGGCAACAAGTACGTCCCTCGCGCCGTCCTCGTCGATCTTGAGCCCGGTA
CCATGGACGCCGTTCGTGCTGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCGT
CTTCGGTCAGTCCGGTGCTGGCAACAACTGGGCCAAGGGTCACTACAACTGAGGGT
A

Fungus identity: *Fusarium solani*

Amplified region: Translation elongation factor sequence

Top hit: Accession number: MG857313

>58-5 F. (726 bp)

CCTCATCGCGATCTGCTTATCTCGGGTCGTGGAACCCCGCCTGGTATCTCGGGCGGG
GTACTCATCAGTCACTTCATGCTGACAATCATCTACAGACCGGTCACTTGATCTACC
AGTGCGCTGGTATCTCCAAGGATGGCAAGAAGTTCGAGAAGGTTGGTCACATCTCCC
CCGATCGCGCCTTGCTATCCCACATCGAATTCCCCGTCGAATTCCCTCCTCCGCGAC
ACGCTCTGCGCCCGCTTCTCCCGAGTCCCAAAAATTTTGCGGTTCGACCGTAATTTTT
TTTGGTGGGGCATTTACCCCGCCACTCGGGCGACGTTGGACAAAGCCCTGATCCCTG
CACACAAAAACACCAAACCCTCTTGGCGCGCATCACGTGGTTCACAACAGACACTG
ACTGGTTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGTTCCTTCAAGTACGCCTG
GGTCCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACCATCGATATTGCTCT
CTGGAAGTTCGAGACTCCCCGCTACTATGTCACCGTCATTGGTATGTCGCCGTCATCT
CTCACTCACGTCTCATCACTAACAATCAACAGACGCCCCCGGCCACCGTGATTTCAT
CAAGAACATGATCACTGGTACTTCCCAGGCCGACTGCGCCATTCTCATCATTGCCGC
CGGTACTGGTGAGTTCGAGGCTGGTATCTCCAAGGATGGCA

>58-5 R. (734 bp)

CTGTAGATGATTGTCAGCATGAAGTGACTGATGAGTACCCCGCCCGAGATACCAGG
CGGGGTTCCACGACCCGAGATAAGCAGATCGCGATGAGGGCTTGACTTACGGTGGT
CGACTTGCCAGAGTCGACGTGGCCGATGACGACAACAGGAGTCTCGAACTTCCAGA
GAGCAATATCGATGGTGATACCACGCTCACGCTCGGCCTTGAGCTTGTCAAGGACCC
AGGCGTACTTGAAGGAACCCTTACCGAGCTCAGCGGCTTCCTATTGTTGAACCAGTC
AGTGTCTGTTGTGAACCACGTGATGCGCGCCAAGAGGGTTTGGTGTTTTTGTGTGCA
GGGATCAGGGCTTTGTCCAACGTCGCCCGAGTGGCGGGGTAAATGCCCCACCAAAA
AAAATTACGGTCGAACCGCAAAATTTTTGGGACTCGGGAGAAGCGGGCGCAGAGCG
TGTCGCGGAGGAGGGAATTCGACGGGGAATTCGATGTGGGATAGCAAGGCGCGATC
GGGGGAGATGTGACCAACCTTCTCGAACTTCTCGATGGTTCGCTTGTCGATACCACC
GCACTGGTAGATCAAGTGACCGGTCTGTAGATGATTGTCAGCATGAAGTGACTGATG
AGTACCCCGCCCGAGATACCAGGCGGGGTTCCACGACCCGAGATAAGCAGATCGCG
ATGAGGGCTTGACTTACGGTGGTCGACTTGCCAGAGTCGACGTGGCCGAAATGACG
A

Fungus identity: *Penicillium citrinum*

Amplified region: ITS sequence

Top hit: Accession number: KX858872

>90-4 F. (503 bp) _merged_90-4 R. (835 bp)

GGCGGGGGGACTTGGCCCAACACACAAGCCGGGCGGGGCAGCAATGACGCTCGGA
CAGGCAGGCCCTCCGAATACCGGAGGCGCAATGTGCGTTCAAGACTCGATGATTCA
CTTAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGG
AACCATGAGATCCGTTGTTGAAAGTTTTAACTAATTTCGTATAGGTCTCAGACTGCA
ACTTCAGACAGCGTTCAGTGGGGCCGTCGGCGGCGCGGGGCCCGCCGAGGCAACAT
AGGTTCGGGCAACACGGGTGGGAGGTTGGGCCCCGAGGGCCCGCACTCGGTAATGA
TCCTTCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCGGGCCCCTCGGGGCCC
AACCTCCCACCCGTGTTGCCCGAACCTATGTTGCCTCGGCGGGCCCCGCGCCCGCCG
ACGGCCCCCCTGAACGCTGTCTGAAGTTGCAGTCTGAGACCTATAACGAAATTAGTT
AAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAAT
GCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACAT
TGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAG
CCCGGCTTGTGTGTTGGGCCCCGTCCCCCCCGCCGGGGGGACGGGCCCGAAAGGCA
GCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTCGTCACCCGCTCTAGTAG
GCCCGGCCGGCGCCAGCCGACCCCCAACCTTTAATTATCTCAGGTTGACCTCGGATC
AGGTAGGGATACCCGCTGAACTTAAGCATATCAAAACGGAGGAA

Fungus identity: *Penicillium citrinum*

Amplified region: β-tubulin sequence

Top hit: Accession number: KC345003

>90-4 F. (439 bp) _merged_90-4 R. (621 bp)

GGGTAAGTAGAACGTTCATGCCCTCCCAGCTGAGATCGGGTTCCCTTGAGCCCCCAA
ATCACCCCCCTTTCTCAAAAAAACTAAACACTTCTTTGTCGAAAGAACTTCTGTCCAT
CGCCATCAAGGCCGTGCTCGCCAGCAATGGTTTGCCTCAGAATTGGTCAGATATTGC
TCCTTGGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATC
AATTGTTGGATACAAAGAGCAATATACTGACCAATTCTATAGGCAAACCATTGCTGG
CGAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGTTTACG
TTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAACCTCCGATCT
CCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATACTGACCCCAATGC
CATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAATAGGCTTCCGGTGACA
AGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGGTACCATGGACGCTGTCC
GTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAACTTCGTTTTCGGTCAATCCGG
TGCTGGTAACAACTGGGCCAAGGGTCAAACTGAGGGTA

Fungus identity: *Penicillium citrinum*

Amplified region: Calmodulin sequence

Top hit: Accession number: AY678555

>90-4 F. (466 bp)

ACTTCAGGCTCACCCAATCTCTTCCTATCGACCGACATTTGCTAAATGGCCACTGTTA
TCTTTGCTGATAGACCCAGGTTGGTTTTGGGGCGGCGATTCCCCCCCTATCCTAACCC
ATCATTCTTTTCTGCTCCTAATTTCAATGTGATCCATATCACCAATCACCACCTCGGA
GCTCGGCACCGCTCTGCCCTCCCTCCCGACCCCTCCAAGTCTGAGATTGATACATGA
TTAACAAGGTTGACGCCCATACCTGGACCATTTCCCCTCTACATCCCATAATCCAGA
TTCTCACATGCCGATTTCCCTTATATTTTTCCGGCCTCGAAGATGAATATTGGCTCGC
CGACCACCTAATTATTAACCATGATGGCCCGAAAGATGAAGGACACTAATACCAAA
TACGAGATTCATTGCATTCTTGGTCTTTGACCGTGATAACTCCTCTTTCATCTCCACT
GCCGAA

>90-4 R. (509 bp)

ACCTTGAATGCCTCCCGAATCTCCTCTTCCGAATCAGGTGTCCTTCATCTTACGGGCC
ATCATGGTGAGGAACTCTGTGGGCCACCCGCCATATTCATCTTCAAAACAGATTGAT
AATAAGGGATATCGAAGTGAGAATCTGGATTATGGGATCTTACCGGGGAAATCATG
GGTGCCTTTGTGATCGGCTTCAACCTCTTTAATCATGTCCTGCACTTCAAACTCGAAG
GGGTTCTGGCCAAGGGAGCGCATGACGGTGCCAACTCCTTGGTGGTGATTTGCCTAT
TGGTAACATTTGTCTCAGTAGGGTTCCAAAAGAATTGGATGGGTTCCGATCGCGGGG
AATCCGCCCGTGAAAATGACACGGGTCTTTCAGCAAAGATGTCTCCCTGTCCTCCTT
GTCCTGTCGATGGCCAATTCCGAGCCTAAAATGTTCACAAATCCACATGCTTCGGTT
CTGGATCACATCACTCCAAAAGGGGAGATGGGCCTCCGTGTACTCGGA

Fungus identity: *Talaromyces ruber*

Amplified region: ITS sequence

Top hit: Accession number: MH856399

>100-2 F. (518 bp) _merged_100-2 R. (632 bp)

CCCGGGACACATCCTCCCCCCGGTCACCCCAGCCCGCCCGCCCCAACCTGTATACAA
CGGGAGAGGTGGCCACGGCCCGCACTCGGTAATATCCTTCCGTAGGGTAACCTGCG
GAAGGATCATTACCGAGTGCGGGCCCTCGTGGCCCAACCTCCCACCCTTGTCTCTAT
ACACCTGTTGCTTTGGCGGGCCCACCGGGGTCACCTGGTCGCCGGGGGACAATCTGT
CCCCGGGCCCGCGCCCGCCGAAGCGCTCTGTGAACCCTGATGAAGATGGGCTGTCT
GAGTACTATGAAAATTGTCAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCCGTGAATCAT
CGAATCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGGCATGCCTGTCCGAG
CGTCATTTCTGCCCTCAAGCACGGCTTGTGTGTTGGGTGCGGTCCCCCCGGGGACCT
GCCCGAAAGGCAGCGGCGACGTCCGTCTGGTCCTCGAGCGTATGGGGCTTTGTCACT
CGCTCGGGAAGGGCTGGCGGGGGTTGGTCACCACCAAAATTTTACCACGGTTGACCT
CGGATCAGGTAGGAGTTACCCGCTGAACTTAAGCATATCATAACCGGAGGAA

Fungus identity: *Talaromyces ruber*

Amplified region: β-tubulin sequence

Top hit: Accession number: LT559076

>100-2 F. (415 bp) _merged_100-2 R. (417 bp)

CCCAGGTGTAACCCAAACGGTGCTGCTTTCTGGTGAGTTTGACTCGACCCAAGCTTA
CTATCAATTGTCGCGACAGCACGCTGACTAGACCAGGCAAATCATCTCTGCTGAGCA
CGGTCTCGATGGCTCTGGTGTGTAAGTATTTCACAATTGGAATACACCTACAGTTCG
ACGATATCTGATCATCGACAGCTACAATGGCTCCTCCGACCTCCAGTTGGAGCGTAT
GAACGTCTACTTCAACGAGGTGCGTTAGAAAGTCTCTCGACTCCTACAGAACAGACA
CTCATTCATCTAGGCCTCCGGCAACAAATATGTCCCTCGTGCCGTCCTCGTCGATTTG
GAGCCCGGTACCATGGACGCCGTCCGCGCTGGTCCCTTTGGTCAGCTCTTCCGTCCC
GACAACTTTGTTTTCGGTCAGTCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTCA
CCTTGAGGGTA

Fungus identity: *Talaromyces ruber*

Amplified region: Calmodulin sequence

Top hit: Accession number: JX315641

>100-2 F. (659 bp) _merged_100-2 R. (452 bp)

TGTTGGCGTGGTTAGCTGACTAGCCGTTTTGATGAATAGGACAAGGATGGAGATGGT
GAGTCGCCGCGAACAATGAAACACCTTGAACGAACATTACCGCAGTCAACAGACAT
TGACCCTATCGGACAGGACAAATCACAACCAAGGAACTCGGCACAGTCATGCGTTC
TCTCGGCCAGAACCCCTCCGAATCCGAATTGCAGGACATGATCAACGAAGTCGACG
CTGACAACAACGGCACAATTGATTTCCCTGGTATGACTGACCACCAACTCGCAATAT
TTGATGGCAGTACTGACTGCCGCAGAATTCTTGACAATGATGGCCCGCAAAATGAA
GGATACCGACTCCGAGGAAGAGATCCGTGAGGCTTTCAAGGTGTTTGACCGTGACA
ACA

Fungus identity: *Trichoderma atroviride*

Amplified region: ITS sequence

Top hit: Accession number: JX119035

>100-14 F. (557 bp) _merged_100-14 R. (637 bp)

GGTGAGACGCACCGGGGCGTGACCCCGCCGAGCACCAGTTTGGTTGTTCACATTGG
GTTTGGAGTTTAACCCGTACCCTTCCGTAGGGTACCTGCGGAGGGATCATTACCGAG
TTTACAACTCCCAAACCCAATGTGAACCATACCAAACTGTTGCCTCGGCGGGGTCAC
GCCCCGGGTGCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGGACCAACCAAAC
TCTTTTCTGTAGTCCCCTCGCGGACGTTATTTCTTACAGCTCTGAGCAAAAATTCAAA
ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAG
CGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC
GCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACC
CTCGAACCCCTCCGGGGGGTCGGCGTTGGGGACCTCGGGAGCCCCTAAGACGGGAT
CCCGGCCCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGC
ACAACTCGCACCGGGAGCGCGGCGCGTCCACGTCCGTAAAACACCCAACTTCTGAA
ATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCATAAACGG
AGGAA

Fungus identity: *Trichoderma atroviride*

Amplified region: Calmodulin sequence

Top hit: Accession number: XM_014086200

>100-14 F. (590 bp) _merged_100-14 R. (467 bp)

CCCCCCCCCCCTATTTGTGCGAACCATTATTGGAGCACCTAAACCTGCGACACCGAT
CAAAATTCCACCGACTGGCAATTTTTATGACGAAGGAACGGGCAAGAAACTAACAG
ACGTGATTTCGCAGGCCAGATCACCACCAAGGAGCTGTCCGAGTACAAGGAGGCCT
TCGATGAGGAAGAGGAGGCGCCAAGGGGCGATGGGGCTGAGGTGGCCGACCTTGA
CAATGAGCCATGCCAGCGTGGATGCGAAGCTTGTCGCGAGGGGTGCGGCTGAAGAA
GATCATGTTASAACGATAAATAGGTGGAGGGAGAGAGAGAYGTACCTGAAATGAAG
ATGGCTGTGCGGAAAGCCAATTCTTGTCTCTTGTAAAAGAAGCTCAAATAGAACGG
AACGCCCGTAAACCCAGCCTCTCCTATGCCCAGCAGCACGCGCAGTGCAATGAGTC
ATCGGGTACGAGACGGCAATGGCTTGCAGCGACGCCGTGATCCCCCACTCATGACC
TCCATCGGGGACATGATCAACGAGGTCAATGCCAACAACAACGGACCCTTCAATTT
CCCTGGTATGTCATTAGCTAAAACACAGGGGACGGCTGCCAAATACGGGCTAATCT
AAAGGGGGGAAAAGTTCCTTACCATGATGGCCCAAAAAATGAAGGACCCTGTTCCA
AAAAGGAAATCCAAAGGTTTTCAAGGTTTGTTATTCCCCCTTGGGCTGTTTTATTTCA
AAACTAATTTGGACAGGTTTTACCCATAACAACGGTTCTTCTCCTGCTGACTGCCCC

Fungus identity: *Trichoderma atroviride*

Amplified region: Translation elongation factor sequence

Top hit: Accession number: KX463439

>100-14 F. (731 bp)

CGGGGTTCCCGGTGTGCAGGGGTCGGTGGTGGTAGTCATCCTACTGAACCCATAACT
CTGAAATGGTCTCGGGCTGCCGTTTGATCTACGGGGAGGTGGGGTTGCAAACCTGCC
AACGGAAATCTTGCCAGGGGGAGCTCATTTCTGCTTTTTGACTCCGCTCCCTGACCG
CGCCCTGCTGTTGCCGGATGACCTGCTTGTCATTTTTTTCCCCATGTGCCGGAACCCC
GCTTGCCTGTCTACCCCACGTTGTTTACGGCAAAATTTTCTGGCTGCCTTGCTTGGCT
TTACGCGGGGTGTCCACTTTTTTTGGTTTGGATGTCTCCCCCCTATCGCCACTGTCCC
GCCCCAACTTGTTGTGCCATTGCATCGTCTTCTCCATCTCTGTGGACTGCATTGCTTG
ATCCGCTTCAGAATATATTAACCCCCGGACATCGTTGGAGGCTCTTACAAATACGTG
GGGGGACTTGAAAGCGTCTGTGACAAAGGTGTATCTGAAAACAATTGGAAACTTTA
TCCTGGAAGTTCTGAACTGGTATCTCAAGGATGGGCATCATTGGTATGTTTTCGCTTT
TCCTCATTGATACTTGGAGACCAAGATTCTAACGTGCCGCTCTGTAGACGCTCCCGG
TCACCGTGATTTCATCAAGAACATGATCACTGGTACTTCCCAGGCTGACTGCGCTAT
CCTGATTATCGCTGCCGGTACTGGTGAGTTCGAGGCTGGTATC

>100-14 R. (690 bp)

GTGGGAAGCTCATGATTCGGCCTTGGTGAGTCACGGCGACCGGGGCGTACAGAGCG
CCGTTAACATTCTGGCGCTCCAATGTTCAAGGAGGAAAGCTCAGACATTGGTACATC
CTACTTGGGAGTCTTGAACTTCCAGAGGGGGTGTCGATGGGATACCACCCTGACGCT
CGGCCTTGCTTGTTAGAACCCCCATACTTGAAAGAACCCTTGCCAAGCTCGGCGCTT
CCTCTTGATTGAAGCCCGGTTCGTGCAGTGCCCCGGAACGATGGATAACATCATGCA
ACTGAGTCCAATTCGTTGGACGGGCCCCGGCGATAAGGGGGTTGCGCCCGCACGAA
AAAATTTCGCTTCCCCGTTTGGAGGCCCACCCTGTCCCAAAAGAAAGCTGGGGCCCC
GAGGGGTAGACAGGTAAAGGGGGGTGATGCTCCGAGGGAAAAAAATGAGCCACTG
AGGACAGATTCAGCAGGAGTGCTGGAGATGATAAAAACCAAAATGGTGTCCCTTCT
TACACAGGTACGCTTGTCAATACCACCGCACTGGTAGATCAAGTGACCAGTCTGTTG
GGAAAATGTCAGCTGGATGCGTTCATGATGATGATAATACCCCGCGCCGACCGACA
AGTCGATGGCTGTAGGGCTCGAGGGATTGGGAGTTACTCACGGTGGTAGACTTGCC
GGAGTCGACGTGC