CHEMICAL GENOMICS: DISCOVERY OF NOVEL FUNGICIDES AND THEIR MODE OF ACTION IN THE PHYTOPATHOGEN *FUSARIUM GRAMINEARUM*.

by

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Abstract

The phytopathogenic fungus *Fusarium graminearum* is the principle cause of Fusarium Head Blight, a devastating disease of wheat, barley and other cereals. A commercial library of chemicals was tested against *F. graminearum*. Of the more than 500 compounds screened, 25 candidates were found to be inhibitory against *F. graminearum* growth. The compound Antofine was tested against a *S. cerevisiae* haploid single knock-out library and 30 mutants were shown to be hypersensitive to this compound. GeneMania, an online multiple association network integration algorithm and the Saccharomyces Genome Database Gene Ontology Term Finder search engine were used to uncover relationships between genes associated with Antofine sensitivity. The results suggested that Antofine likely perturbs genes involved in transcription regulation and mRNA processing.

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List of Abbreviations

ACN – Acetonitrile (CH_3CN)

AUC – Area Under the Curve

$CHCl_3$ – Chloroform

CV – Coefficent of Variation

DMSO – Dimethyl sulfoxide

DON – Deoxynivalenol

EtOAc – Ethyl Acetate ($CH_3COOCH_2CH_3$)

FHB – Fusarium Head Blight

GFP – Green Fluorescent Protein

IC_{50} – Half Maximal Inhibitory Concentration

MeOH – Methanol

NGV – Normalised Growth Value

TLC – Thin Layer Chromatography

YPD – Yeast Peptone Dextrose

ZON – Zearalenone

Chapter 1 Introduction

1.1 *Fusarium graminearum*: A phytopathogen of cereals

1.1.1 *F. graminearum*, a brief overview

F. graminearum Schwabe [telemorph *Gibberella zeae (Schweinitz)* Petch] is a phytopathogenic fungus that was first reported in England in 1884, and is currently the subject of intensive study (Trail, 2009). The fungus is classified as an ascomycete (Phylum - Ascomycota) under the class Sordariomycetes and the order Hypocreales in the family Nectriaceae (Kendrik, 2000).

F. graminearum is a necrotrophic pathogen of a number of economically important cereal crops; principally wheat (*Triticum aestivum* L.), oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), and corn (*Zea mays* L.) Strains of *F. graminearum* have been isolated and recognized worldwide as the primary causal agent of Fusarium Head Blight (FHB), in North and South America, Europe, Australia, and Asia, (Desjardins and Proctor, 2007; Trail, 2009).

Several other *Fusarium* species, (*F. culmorum*, *F. poae*, *F. pseudograminearum*, and *F. avenaceum*) have also been associated with kernel blight seedling blight, brown foot rot, head blight, scab, and ear and stalk rot and other related diseases, with individual species predominating in specific grain hosts, regions of the world, or under certain climatic conditions (Goswami and Kistler, 2004; Trail, 2009; Yuen and Schoneweis, 2007).

The *F. graminearum* genome is composed of four chromosomes comprising a genome of about 36.1 Mb, which is a typical size for a filamentous fungus; however most related fungi have 9 to more than 17 chromosomes, suggesting chromosome fusions occurred in the distant past (Cuomo *et al*, 2007; Trail, 2009). In May of 2003 the complete genome of *F. graminearum* was released by the Broad Institute/MIT Center for Genome Research, and is undergoing annotation through the efforts of many researchers, the most notable of which is MIPS (Munich Information Center for Protein Sequences) (Goswami and Kistler, 2004; Desjardins and Proctor, 2007).

1.1.2 Overview of disease life cycle

1.1.2.1 Overwintering

F. graminearum is homothallic (self-fertile), granting it the ability to reproduce with itself without the necessity of outcrossing, however it spends the majority of its life cycle in a haploid state (Cuomo *et al*, 2007; Trail, 2009). Sexual development and the eventual production of ascospores begin with the development of binucleated (two nuclei) hyphae, called dikarya (Trail, 2009). A dikaryon forms a small coiled cell that is the predecessor to an ephemeral sexual structure called a perithecium, from which ascospores are generated through meiosis (Trail, 2009). Tubular sacs called Asci (*s.* Ascus) containing eight developing ascospores each are produced within the perithecia. When mature, the perithecia are transferred to the outer opening of the fruiting body and induced to discharge their ascospore cargo into the air through turgor pressure generated propulsive force, where the spores are dispersed by wind, rain or animals. (Trail *et al*, 2002; Trail, 2009).

F. graminearum can also spread shorter distances via water splash through the formation of conidia; non-motile asexual spores produced through mitosis and generated from hypha via specialized stalks called conidiophores. The formation of conidia is often observed on the surface of infected plants and crop residues during periods of extended dampness and rainfall (Trail, 2009).

Ascospores are considered to be the primary inoculum for most *Fusarium* infestations since they are airborne and are the variety of spore most often observed. (Shaner, 2003; Trail, 2009; Trail *et al*, 2002). Ascospore release is dependent upon light and moisture conditions, and often corresponds with anthesis of host plants in the spring and early summer (Trail *et al*, 2002). Host plants are most vulnerable to infection from the flowering and pollination period up to the soft dough stage of kernel development (Bai and Shaner, 1994; McMullen *et al*, 1997).

The airborne spores land on a flowering spikelet, germinate and the hyphae or vegetative form of the fungus, invades the plant tissues through natural openings such as the stoma, the base of the lemma and palea, or through the soft degenerating tissues of the anther. (McMullen *et al*, 1997; Trail, 2009). Once the fungus has penetrated the host the developing hyphae spread throughout the apoplast and cortex of the plant, migrating away from the infected spikelet and towards the rachis. Arriving at the rachis the invading organism utilises the vasculature to spread radially to colonise the rest of the plant (Guenther and Trail, 2005: Trail, 2009).

Initially the growth is asymptomatic, but almost immediately after penetration the fungus will activate metabolic pathways to generate mycotoxins and cell wall digesting enzymes causing necrosis. This is followed by bleaching of the effected tissues, a typical indication of head blight in wheat (Trail, 2009).

Developing seeds colonized by the invading fungus shrivel to form lightweight undersized kernels often referred to as tombstones (Trail, 2009). Alternatively, the clogging presence of the mycelium in the vascular tissues can prevent adequate water and nutrients from reaching other developing kernels, causing the head to ripen prematurely, or even prevent kernel development entirely (Bai and Shaner, 1994).

Eventually the plant succumbs to the infection, and once its host is dead the fungus continues with its life cycle to form conidia or new perithecia so that it can spread and colonise new plants or plant debris. In infected wheat plants, the development of perithecium is often observed to be associated with the plant's stomates and silica cells (Guenther and Trail, 2005). The extensive colonisation of the wheat stems contribute to disease incidence in the field, since these tissues often remain intact over the winter to provide a source of inoculum the following spring (Dill-Macky and Jones, 2000). A new seasonal cycle of infection will begin with *F. graminearum* overwintering as perithecia and dikaryons in contaminated soil, grass and debris from the previous harvest, or being introduced through sowing of contaminated seed. (Goswami and Kistler, 2004; Guenther and Trail, 2005).

1.1.3 Health and economic implications of *F. graminearum*

1.1.3.1 Health

The most significant portion of the damage that members of the *Fusarium* genus cause during infection, in addition to physical harm, is the production of mycotoxins; which in sufficient quantities renders the contaminated grain unfit for consumption by humans or livestock (Trail, 2009). Three mycotoxin types (trichothecenes, zearalenones and fumonisins) have proven to be the principal cause of animal sickness, while the remainder (beauvericin and enniatins, equisetin, fusarins) have been demonstrated to be toxic or carcinogenic under laboratory conditions (Desjardins and Proctor, 2007; Trail, 2009).

The primary mycotoxin produced by *F. graminearum* is deoxynivalenol (DON) sometimes called vomitoxin, a potent protein biosynthesis inhibitor that binds to peptidyl transferase, an integral part of the 60S ribosomal subunit (Desjardins and Proctor, 2007; McMullen *et al*, 1997; Trail, 2009; Yazar and Omurtag, 2008). DON and other members of the trichothecene group are the mycotoxins most often associated with chronic and fatal toxicoses of humans and animals from consumption of contaminated grains (Desjardins and Proctor, 2007; Yazar and Omurtag, 2008). The toxin has been implicated as the likely causal agent of Alimentary Toxic Aleukia reported in Russia during the 19th century; outbreaks of akakabi-byo (red mold disease) in Asia caused by infected wheat and barley during the 1930's to 1970's, and swine feed refusal and estrogenic syndrome in the United States, caused by tainted corn between 1920-1980 (McMullen *et al*, 1997; Yazar and Omurtag, 2008).

Another mycotoxin of concern is zearalenone (ZON), which although not identified as acutely toxic, has been demonstrated to have estrogenic effects in swine and experimental animals which is of particular concern in animal husbandry (Desjardins and Proctor, 2007; Guenther and Trail, 2005; Trail, 2009).

Production of fumonisins, although not typically attributed specifically to *F. graminearum*, are important mycotoxins in other *Fusarium* species (Yazar and Omurtag, 2008). Fumonisins act through the inhibition of sphingolipid metabolism, and were implicated in the 1970 outbreak of equine leukoencephalomalacia (fatal brain lesions) in horses after consumption of tainted feed in South Africa, and as the cause of fatal porcine pulmonary edema in pigs fed contaminated corn screenings from the 1989 corn crop in Iowa, Illinois, and Georgia (Yazar and Omurtag, 2008). Fumosins are also carcinogenic and have been associated with human esophageal cancer and birth defects (Desjardins and Proctor, 2007).

The decontamination of mycotoxin tainted grains is difficult due to compound stability under most temperature and pH conditions employed during grain processing and food preparation (Yazar and Omurtag, 2008).

1.1.3.2 Economics

Damage from FHB is multifaceted; infection results in reduced yields and test weights, while mycotoxin contamination reduces both quantity and quality of grain and seed (McMullen *et al,* 1997). Because of the prevalence of host species, *Fusarium* infection and subsequent mycotoxin contamination is all but impossible to completely prevent in the field. Producers who discover high rates of blight in their fields are often confronted with the difficult decision of spending the time and resources to salvage what could prove to be an unsalable crop, or to destroy the crop at the expense of the time and resources that have already been spent (McMullen *et al*, 1997).

In combination with the difficulties associated with mycotoxin decontamination during post-harvest, most countries now follow the policy of standardised testing and the establishment of maximal allowable limits in foodstuffs sold for both human and animal consumption (Egmond and Jonker, 2004). Grain is tested and then classified according to grain quality and mycotoxin level; grain graded as low quality can be mixed with grain graded to be of especially high quality to make the mixture salable (Windels, 2000). However if large portions of harvested grain are contaminated it will have profound effects upon the economy of the grain market. It is not uncommon for contaminated shipments of grain to be refused for import or purchase (McMullen *et al*, 1997).

The United States Department of Agriculture has classified the re-emergence of *Fusarium* head blight as the worst plant disease epidemic since the stem rust epidemics of the 1950s (Wood *et al*, 1999). During a survey in 1917 wheat losses from *F. graminearum* infection in the United States were recorded to 288,000 metric tonnes, (10.6 million bushels), and in 1919 to be 2.18 million metric tonnes (80 million bushels)

(McMullen *et al*, 1997: Windels, 2000). Cool moist weather in 1982 produced yet another epidemic resulting in a loss of 2.72 million metric tonnes (100 million bushels) of wheat for an estimated 4% reduction in total United States wheat production (McMullen *et al*, 1997: Windels, 2000).

During the 1990's, twenty six American states and four Canadian provinces reported *Fusarium* epidemics of varying severity (Windels, 2000). Over 500 million bushels of wheat were reported lost in the USA at an estimated value of more than $2.5 billion USD (Windels, 2000). Barley producers were less affected, but reported losses greater than $400 million USD, bringing the total loss of revenue between the two cereals to be close to $3 billion USD (McMullen *et al*, 1997: Windels, 2000). During the 1990s, wheat producers in the Canadian provinces of Ontario and Quebec estimated their losses to be $220 million USD, and when disease severity reached 20-80% between 1993 and 1998, an estimated loss of $300 million USD was recorded in the province of Manitoba alone (Windels, 2000).

1.2 Fusarium control methods

1.2.1 Agricultural practices

The most efficient and cost effective method of controlling the damage caused by *F. graminearum,* is through integrated pest management. Integrated pest management involves establishing acceptable pest levels, closely monitoring pest presence, and using preventative cultural practices, mechanical controls, and responsible use of chemical pesticides to keep pest presence to an acceptable level. (USEPA, 2012).

This begins with the use of seed that has been certified to be disease free, or has been treated with a pesticide to inhibit infection during the early stages of growth. Although seed treatment is unlikely to be of any help with spikelet infection, it would at least prevent the introduction of inoculum from external sources (Teich and Nelson, 1984). As an added benefit, treated seed has also been associated with high seedling vigour (Bai and Shaner, 1994).

Proper maintenance and cleaning of equipment is also important so that a problem in one area is not transferred to another. The use of dirty or contaminated equipment (contaminated seed drills for example) on multiple fields without proper cleaning and sanitation has the potential to spread disease from a contaminated field to a healthy one. Seeding by air is one potential solution to this problem, but has other disadvantages (Teich and Nelson, 1984).

Another good agricultural practice is adequate fertilisation to provide the best chance for the young plants to grow strong and healthy, and the application of pesticides to help control FHB and other pests. When farmers are forced to adjust to losses, they often apply less fertiliser and pesticides, and skimp on equipment maintenance (Windels, 2000). Ironically these practices would only make the problem worse, since nutrient stress is associated with greater susceptibility to infection (Bai and Shaner, 1994). Unfertilised plants are often less robust and able to fend off disease, while a reduction in pesticide applications will likely increase the reservoir of inoculum or provide the opportunity for other pests to weaken the plants making them more susceptible. In a survey performed in Lambton County of Southwestern Ontario, Canada in 1983, a significantly higher incidence of disease was noted in fields with lower than

the recommended 90 kg/ha nitrogen, or medium to low quantities of phosphorus present (Teich and Nelson, 1984).

The removal of unwanted plant species in the area serves a dual purpose. Weeds in a field could either be a source of stress by reducing nutrient or water availability, or if the species will support *Fusarium* growth, by serving as a source of inoculum (Bai and Shaner, 1994; Teich and Nelson, 1984). In the aforementioned Lambton County survey, the presence of noticeable amounts of weeds (mainly quack grass, ragweed, buckwheat and mustards) was correlated with a doubling of measured blight in comparison with weed-free fields (Teich and Nelson, 1984).

Prompt harvesting at maturity reduces the time in which spores can land upon the plants, allowing the fungus to invade mature kernels or plants. In addition, *Fusarium* is known to increase the production of mycotoxins if a crop ripens but harvest is delayed due to wet weather (Purchase, 2007). Proper storage of harvested material by harvesting or drying it to a moisture content of less than 14%, storage in a cool and dry environment, removal of possible sources of inoculum such as tombstone kernels, is critical to maintain the quality and salability of a crop (Purchase, 2007).

1.2.2 Crop rotation

Reduction in fungal inoculum from year to year is critical for reducing the risk of severe FHB infections (McMullen *et al*, 1997). Planting alternative crops that cannot support *Fusarium* growth, and rotating through a variety of species is one method of both interrupting the pathogen's life cycle and enriching the soil (Windels, 2000). Current agricultural practice however, favours dense cereals monocultures, with little to no crop rotation; or favour crop rotations with few plant varieties rotated in very short cycles

providing the opportunity for inoculum to build up to epidemic levels (McMullen *et al*, 1997; Windels, 2000). Corn residues in particular appear to provide the greatest amount of overwintering inoculum in comparison with cereals, which is believed to be attributed to either slower crop residue degradation, or greater provision of inoculum due to mass. Planting wheat or other cereals immediately after corn should be avoided if at all possible (Teich and Nelson, 1984).

1.2.3 Removal or tillage of crop residue into the soil

Tillage, or the mixing and rotation of top soil in preparation for planting is a means to bury most of the residues of the previous plantings and is highly recommended as a method of reducing the sources of FHB inoculum (Bai and Shaner, 1994; Windels, 2000). Chaff and light-weight tombstone kernels and other infected head debris are often returned to the soil surface during the harvest process, providing an overwintering site for the fungus (McMullen *et al*, 1997). Tillage of the soil helps to bury such harvest detritus and reduce the number of existing sources of inoculum. Disturbing the soil in this manner however, comes at the cost of drying the soil before seeding, reducing the infiltration rate of water causing increased run-off, eutrophication and hastens organic decomposition and disappearance, thereby reducing the available quantities of nutrients (Daum, 1996). Tillage also increases the rate of soil erosion, decreases the quantity of desired organic matter in the soil (Microbes, earthworms, ants etc.), will compact the subsurface soil, and can potentially attract unwanted organisms to the field (Daum, 1996). For these reasons, and for the savings in money and time, the adoption of "Reduced tillage" or "Conservation Tillage" methods has started to become prevalent; a practice that has favoured inoculum survival (Windels, 2000).

1.2.4 Selective breeding for resistance

Selective breeding is the process by which a plant breeder attempts to "improve" a particular cultivar of plant through the repeated selection of breeding pairs on the basis of desirable traits such as; improved yield, appealing taste, or large fruit. Alternatively, established cultivars can be bred with wild varieties in the hopes of introducing or reintroducing new and desired traits, such as drought or salt tolerance, improved vigour, or increased disease resistance into that cultivar (McCouch, 2004). Many of the major crops consumed today derive from almost inedible ancestors which had poor yields or were difficult to harvest; but through more than 10 000 years of selective breeding by humans, were modified to become better suited for efficient and dense agriculture (McCouch, 2004). This selection of desired traits can often come at the expense of others; an increase in seed size and quantity may come at the expense of a loss in overall plant resiliency against poorer growing conditions, or a reduction in the ability to resist disease (McCouch, 2004). For example, during the aftermath of the 1993 *Fusarium* epidemic, many of the agricultural industry and government professionals collaborated to screen existing wheat and barley cultivars along with other grass species for disease resistance in combination with desirable physical traits (McMullen *et al*, 1997). Less than 1% of the cultivars and crosses screened demonstrated any resistance to FHB; this 1% has become the focus of selective breeding so that these resistance qualities can be incorporated into the cultivars currently adopted for acceptable yield and quality characteristics.

Selective breeding for resistance to *F. graminearum* has been observed to be of only limited success in wheat under heavy disease pressure; since heavy infestations will often simply overwhelm the plants' defences by sheer pathogenic loading (Trail, 2009).

1.2.5 Chemical pesticides to control Fusarium

The application of broad-spectrum pesticides with adequate coverage, the cost associated with spraying, and the difficulty in determining the optimal time to apply pesticides is both problematic and expensive (Trail, 2009). In Canada, there are few active ingredients registered to suppress *F. graminearum* as a foliar or seed treatment in cereals. Five of these, tebuconazole, prothioconazole, triticonazole, difenoconazole, and ipconazole belong to the triazole group of fungicides, and work by inhibiting sterol biosynthesis. The triazoles, in addition to chlorothalonil, encompasses the majority of the fungicides registered for foliar control of *F. graminearum* in cereals. (Health Canada, 2012; FRAC, 2011). Three others; chlorothalonil (a chloronitrile), maneb, and thiram (dithiocarbamates), are all classified as having multiple sites of action. Metalaxyl is an acylalanine that acts upon RNA polymerase, pyraclostrobin is a methoxycarbamate that inhibits cellular respiration by inhibition of complex III of cytochrome bc1 (ubiquinol oxidase), and fludioxonil is a phenylpyrole that acts upon osmotic signal transduction. Most of these fungicides are only available as seed treatments (Health Canada, 2012; FRAC, 2011). Because of the ubiquitous global distribution of *F. graminearum*, all of these techniques offer only moderate control, but not eliminate the proliferation and damage the disease causes, and the realised gains of employing them must be balanced against the costs and time involved (Guenther and Trail, 2005).

1.3 Use of *S. cerevisiae* to decipher mode of action of chemical fungicides

Saccharomyces cerevisiae has proven to be a robust system to study the function and effects of chemicals/drugs on cellular processes (Kumar and Snyder, 2001). The organism is economical and easy to grow; the cell membrane is highly permeable to small molecules, and *S. cerevisiae* has a genome of manageable size that can be easily manipulated (Bharucha and Kumar, 2007). The genome of *S. cerevisiae* has been sequenced, and many of the genes either have been, or are in the process of being annotated (Mager and Winderickx, 2005; Giaever *et al*, 2002; Winzeler *et al*, 1999). But most importantly, many of the cell processes in yeast are conserved in higher eukaryotes (Melese and Hieter, 2002). *S. cerevisiae* has become a choice eukaryotic model for the development of genomic technologies, and there are many resources that have become commercially obtainable. For example, yeast deletion libraries are available in which nearly all ~6000 known and suspected Open Reading Frames (ORF) have been replaced with a selectable marker gene such as *KanMX* (Geneticin resistance), bracketed by unique molecular "barcodes" 20-nucleotides long (Giaever *et al*, 2002).

One method of using a yeast deletion library is called Homozygous profiling (HOP) in which the fitness of an array of haploid deletion mutants is assessed in the presence of a chemical compound (Costanzo, 2006). The basic principle is that since a haploid organism only has a single copy of each gene, then deleting one gene disrupts all of the processes in which that particular gene product participates. The disruption of a particular process is not always fatal, since not all processes are essential and there are many cases of pathway redundancy (Winzeler *et al*, 1999). A simple example is that of two separate but redundant pathways for an essential process. The deletion of a gene

disrupts one pathway forcing the organism to rely upon the alternative pathway. If a chemical compound disrupts one of the components of the alternative pathway, then the combination of the deletion and drug results in greatly reduced growth, or even lethality (Winzeler *et al*, 1999). By examination of the interrelationships between the deleted ORFs of the sensitive strains, it becomes possible to determine which pathways and processes are directly affected by that drug. Another similar method is Haploinsufficiency profiling (HIP) which is essentially the same process, but instead uses heterozygous yeast deletion mutants which, by virtue of having representatives of both essential and non-essential genes, enables the direct identification of cellular targets (Costanzo, 2006).

1.4 *Vincetoxicum rossicum* (Kleopow) Barbar – A source to control Fusarium

The invasive herbaceous perennial *Vincetoxicum rossicum* (Kleopow) Barbar. [syn: Cynanchum rossicum (Kleopow) Borhidi] commonly known as pale swallow-wort, or the dog strangling vine was introduced to North America from European Russia and Ukraine in the late 1800s (Sheeley and Raynal, 1996; Pringle, 1973). Likely imported originally as an ornamental species, major infestations of the invasive plant can now be found in the Canadian provinces of British Columbia, Ontario and Quebec, and in nine US states (DiTommaso *et al*, 2005; USDA, 2012). A member of the milkweed family (Apocynaceae subfamily Asclepiadoideae), *Vincetoxicum spp.* as well as members of the related genus *Tylophora*, have been shown to produce phenanthroindolizidine alkaloids (Staerk *et al*, 2005). In previous experiments the phenanthroindolizidine alkaloid Antofine (Figure 1) was isolated from *Vincetoxicum rossicum*, and observed to have inhibitory effects against a variety of microorganisms, including two strains of

F. graminearum (Mogg *et al*, 2008). Although treatment with Antofine has been demonstrated to inhibit protein, DNA and RNA synthesis, the exact mode of action has yet to be discovered (Wang *et al*, 2010; Gibson *et al*, 2011).

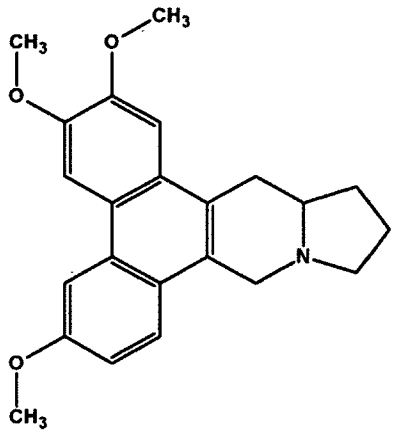

Figure 1: Chemical Structure of Antofine, a phenanthroindolizidine alkaloid.

1.5 Thesis outline

In addition to good agricultural practices, current integrated pest management strategies to control *F. graminearum* include application of chemical fungicides. However, with the emergence of Fusarium strains resistant to common fungicides and the environmental impact of their use, there is a need for the development of new and effective fungicides. The overall goal of my thesis was the discovery of novel fungicides and the investigation of their mode of action.

To fulfill the first objective of my thesis a natural product derivative library composed of compounds derived from natural sources was screened in liquid culture against *F. graminearum* to uncover those that perturbed the organisms growth (Section 2.2); several molecules of interest were identified. To fulfill the second objective a haploid *S. cerevisiae* mutant library was used to identify potential targets of these compounds. The screening of the yeast mutant library is presented in Section 2.5. The use of this heterologous system enabled me to uncover mechanism(s) of action of these compounds in *F. graminearum*.

Chapter 2 Materials and Methods

2.1 *F. graminearum* and *S. cerevisiae* strains used in this study

2.1.1 Fusarium strains, propagation and storage

The strain *F. graminearum* ZTE-2A (also catalogued as DOAM # 227650) is a transformant of *F. graminearum* wild type (DOAM # 233423) in which GFP (Green Fluorescent Protein) is constitutively expressed (Van den Wymelenber *et al*, 1997; Miller *et al*, 2004). The GFP fluorescence provides a method by which mycelia biomass can be accurately quantified by mechanical and automated means.

For storage purposes, conidia were generated using carboxymethylcellulose (CMC) growth medium (see appendix for detailed composition) according to Cappellini and Peterson (1965), and were suspended in 15% glycerol and stored at -80°C. This stock was used to prepare fresh conidial stock for use as the primary inoculum in subsequent experiments. The strain was retrieved from, and continues to be maintained as part of the Canadian Collection of Fungal Cultures by Agriculture and Agri-Food Canada.

2.1.2 Maintenance and storage of *S. cerevisiae* strains

S. cerevisiae strain BY4741 was used as the wild type strain for all growth comparisons and inhibitory studies (YSC1048, Open Biosystems (https://www.openbiosystems.com). The strain is haploid mating type designated "Mat a" and is the parental strain from which the Yeast Knock Out Collection used in subsequent experiments was derived. The diploid *S. cerevisiae* strain BY4743, (YSC1050, Open Biosystems) was used in some experiments.

For long term storage purposes, cells were propagated in YPD (Yeast Extract Peptone Dextrose) at 30°C shaking at 200 RPM overnight. Cells were diluted to $OD_{600} = 0.5$, incubated for 2 hours, and suspended in 15% glycerol and stored at -80°C. The frozen culture was used as a source for a solid streak culture on 2% YPD_{AGAR} which was incubated for 24 hours at 30°C and then stored at 4°C for periods not exceeding one month. Single colonies from this streak culture were used as a source of inoculum for routine work.

2.1.2.1 The *S. cerevisiae* deletion or knock-out library

The "Yeast Knock Out MATa Collection" used for the screening experiments was purchased from Open Biosystems catalogue # YSC1053. It is a haploid mating type designated "Mat a" and is provided frozen in glycerol in 96 well plates. The collection was kindly provided by the lab of Dr. Steven Gleddie of Agriculture and Agrifoods Canada, Ottawa, Ontario.

The library was copied to solid 2% YPD agar media contained in an Omniwell plate (Cat # 242811, NUNC) using a 96 pin replicator (Cat # 250520, NUNC) and the OmniTray Copier (Cat # 250555, NUNC) apparatus to condense the library into plates at ~ 384 colonies per plate. Thereafter the library was transferred or copied using a 384-Pin Replicator (Cat # 250393, NUNC) and the Microwell® Plate Copier (Cat # 250539, NUNC). The collection was maintained in solid form at 4°C, and to revitalise the cells before each experiment, the library was transferred to fresh 2% YPD agar, and incubated overnight at 30°C.

The library replication method was used in accordance with the NUNC™ Replication System. Briefly, a sterile 384-Pin Replicator is used to inoculate the receiving plate from the source plate using the appropriate index holes in the OmniTray Copier apparatus. The 384-Pin Replicator is sterilised between different source plates by sequentially dipping the pins in reservoirs containing; a 10% bleach solution, followed by a sterile distilled water rinse, and finally 95% alcohol. The replicator was then flamed briefly with a Bunsen burner, and allowed to cool for 1-2 minutes before being used to copy the next source plate in the library.

2.2 High-throughput screen of *F. graminearum* against a chemical library

The chemical library NDL-3000 used in this study was purchased from TimTec (http://www.timtec.net/) a provider of chemical compound collections for screening and analytical purposes. The compounds are supplied in 96 well plates as 50 µL stock solutions dissolved at 2 mg/mL in DMSO (dimethyl sulfoxide).

A selection of crude plant extracts provided by Andrew Waye, at the University of Ottawa included wood, bark and leaf tissue collected in September 2007 near Mistissini Quebec from common juniper [*Juniperus communis* L. {voucher # AW03-W01}], white spruce [*Picea glauca* (Moench) Voss {voucher # AW04-B01}], black spruce [*Picea mariana* (Mill.) BSP {voucher # AW02-B01}], balsam fir [*Abies balsamea* (L.) Mill. {voucher # AW01-W01}], and jack pine [*Pinus banksiana* Lamb. {voucher # AW06-B01}]. Also included were samples collected in 2008 near Denholm Quebec from eastern hemlock [*Tsuga canadensis* (L.) *Carr.* {voucher # AW09-W01}], tamarack larch [*Larix larcina* (Du Roi) Koch {voucher # AW05-W01}], white cedar [*Thuja occidentalis* L. {voucher # AW10-W01}], and white pine

[*Pinus strobus* L. {voucher # AW11-W01}]. Voucher specimens of all the aforementioned plants are retained at The University of Ottawa, in Ottawa, Canada.

The tissues were extracted using methanol (MeOH), ethanol (EthOH), or ethyl acetate (EtOAc), dried by a combination of roto-evaporation and vacuum desiccation, and provided as dry solid material. The crude extracts were suspended in DMSO and used at a stock concentration of 200 mg/mL.

Tests were performed in triplicate in a 96 well fluorescent assay plate, containing 800 spores of *F. graminearum* (ZTE-2A), suspended in 200 µL of GYEP, with 0.07 mg/mL of test compound in 3.5% DMSO. Each plate included wells with 800 spores in GYEP with 3.5% DMSO alone to serve as positive controls, and with the two antibiotics, Hygromycin – B ™ and Geneticin ® dissolved at 0.150 mg/mL to serve as positive controls. The plate was sealed with parafilm and incubated at 28°C, shaking at 300 rpm. Growth was evaluated in 23 minute intervals over 72 hours using a BMG Labtech POLARstar OPTIMA microplate fluorimeter, via 485 nm excitation and 520 nm emission filters, with gain adjusted to 10% maximum of control wells.

2.3 Statistical analysis of growth rates

2.3.1 Analysis of *F. graminearum* growth rates

The chemical effect upon *F. graminearum* growth rates was analysed as follows: To compensate for effect of compounds absorbing or fluorescing at 520 nm, a baseline correction was performed by subtracting the smallest value in each data set from all other values within that dataset. Each dataset was then integrated using the MARS Data Analysis Software Package (Ver. 2.10R3), provided with the plate reader, to calculate the

area under the curve (AUC) between 0 and 72 hours. The significance of each sample mean in comparison with the control was tested using a two-tailed unpaired t-test for two samples assuming an equal variance; samples for which a *p*-value less than 0.05 were considered to be significant.

Efficacy of each compound was assessed by normalizing the average area under the curve (AUC) from the test compound with respect to control.

$$NGV = \frac{Average\ AUC_{Test}}{Average\ AUC_{Control}}$$

Where NGV = Normalised Growth Value, AUC = Area Under the Curve

Any compound with a normalised growth value (NGV) greater than 1.75 ($p < 0.05$) was considered to be an enhancer of growth; an NGV value between 0.75 and 0.1 ($p < 0.05$) was considered to be a growth suppressor; and an NGV value less than 0.1 ($p < 0.05$) was considered indicative of a inhibitor at the concentration tested (0.07 mg/mL).

The Coefficient of Variation (CV) was calculated as follows:

$$CV = \frac{Standard\ Deviation\ of\ Sample\ Replicates}{Average\ of\ Sample\ Replicates}$$

2.4 Isolation and purification of Antofine

The procedure used for the isolation and purification of Antofine was adapted from the methods employed by Mogg *et al* (2008), and the flow chart outlining the method is shown in Appendix Figure 1. Briefly, root tissues of *Vincetoxium rossicum* were harvested, vacuum desiccated and crushed in EtOAc. Voucher specimens (#2005-10) of *V. rossicum* are retained at Carleton University, Ottawa, Canada.

The crude extract was filtered, concentrated by rotary evaporator, and dissolved in 0.01 M HCl_{aq}. The pH was reduced to 2.0 with 1M HCl_{aq} and the solution partitioned with EtOAc. The organic fraction was concentrated by rotary evaporator and dried for storage. The pH of the aqueous fraction was increased to 10 with 1M NH_4OH_{aq} and partitioned with EtOAc. The alkaline fraction containing Antofine was concentrated by rotary evaporator, dissolved in pure acetonitrile (ACN) and injected into a 250 x 10mm 5 μm 120Å C18 reverse phase semi-preparatory column (ACE-121-2510, Advanced Chromatography Technologies). The column was washed in a gradient (5% ACN in water to 75% ACN in water) over 20 minutes at 10 mL/min. Antofine was eluted in 75% ACN in dH_2O in 10 mL fractions for 20 minutes. A 2 mL aliquot of each sample was dried using a speed vac (SVC-100H, Savant), and suspended in 200 μL MeOH. A 10 μL aliquot of the suspension was spotted onto a sterile 6 mm filter disc. The discs were placed amended side down on petri dishes containing 2% YPD_{AGAR} evenly spread with ~ 1.0×10^6 CFU *S. cerevisiae* BY4741, and incubated at 30°C overnight. The fractions which demonstrated an inhibition zone were pooled and dried using a rotary evaporator.

The pellet was suspended in MeOH and spotted onto a glass backed silica Thin Layer Chromatography (TLC) plate (5715-7 - EMD Chemicals). Compounds were separated by TLC with two consecutive migrations of 60:40 EtOAc:MeOH, and traced by illumination under short wave (254 nm) UV light. Each band was scraped from the silica plate and recovered using MeOH. The MeOH was removed by rotary evaporator, and the recovered compounds was solubilized in $CHCl_3$, and tested for activity as previously described.

The isolate with Antofine activity was subjected to ^1H NMR for confirmation. The NMR spectrum was recorded on a Bruker Advance 400MHz NMR Spectrometer. The sample was recorded using $CDCl_3$ as the solvent, purchased from Cambridge Isotope Laboratories Inc. The reference peak was set using the known residual solvent peak of $CDCl_3$ at 7.26ppm.

2.5 Antofine-genetic interactions in *S. cerevisiae*.

To determine the appropriate sub-lethal concentration of Antofine to be used to screen the "Yeast Knock Out MATa Collection" (YSC1053, Open Biosystems) a small volume test was performed to test five concentrations simultaneously in a six well plate. The method employed is an adaptation of the agar dilution method detailed by (Wiegand *et al*, 2008). Briefly, aliquots of Antofine were dissolved in 500 µL of YPD_{BROTH} to form solutions at four times the desired final concentration (for example, for a final concentration of 0.40 µg/mL, a broth concentration of 1.60 µg/mL was made). The solutions were transferred to a 6 well plate (3046, Falcon), placed on a heat block set to 55°C to warm. The heat block and plate combination were placed onto a table top orbital shaker and allowed to mix at low speed for 5 minutes. To each solution was added

1.5 mL of molten 2% YPD_{AGAR} cooled to 55°C, bringing the final volume in each well to 2 mL, and reducing Antofine to the desired concentration (in this example 1.60 µg/mL becomes 0.40 µg/mL) in 1.5% Agar. The plate was allowed to mix for 5 minutes before the plate was removed from the heat block and placed in a laminar flow cabinet to set. The solid Agar was allowed to solidify for at least 30 minutes before inoculation.

Two ten-fold dilutions in YPD_{Broth} of *S. cerevisiae* BY4741 (haploid) and BY4743 (diploid) from OD_{600} = 0.1 (~1.5×10^6 CFU/mL) to OD600 = 0.0001 (~1.5×10^3 CFU/mL) were spotted twice in 2 µL aliquots onto the media within each of the wells of the prepared assay plate. The liquid was allowed to be adsorbed before the plate was sealed with parafilm and incubated inverted at 30°C. Plates were examined and observations recorded every 24 hours.

2.5.1 Screening Antofine against the haploid library in solid media

Based upon the results of the small volume experiments using the 6 well plates, the entire Yeast Knock Out MATa Collection library containing a total of 4847 mutants spread over 14 plates at 376 individual colonies per plate, was screened using 0.20 µg/mL of Antofine in 1.5% YPD_{AGAR}. The colonies were transferred to the experimental and control plates by the method outlined in section 2.1.2.1, and growth of the colonies was recorded for three days and analysed using the ScreenMill program (Section 2.6).

Of the entire library of 4847 mutants, 232 (p value <0.05) mutants were selected for retesting and spotted in quadruplicate over three YPD_{AGAR} plates. This sub library was screened with a range of Antofine concentrations from 0.20 µg/mL to 0.60 µg/mL in 0.05 µg increments and from 1.5 µg/mL to 4.5 µg/mL in 1 µg/mL increments.

2.6 ScreenMill analysis

The analysis of the resultant differences in colony growth was performed using the ScreenMill growth measurement and analysis Software Suite (Dittmar *et al*, 2010). The ScreenMill software suite consists of three software tools: The Colony Measurement Engine (CM Engine), The Data Review Engine (DR engine) and the Statistics Visualisation Engine (SV Engine). The CM Engine is an open source macro for *ImageJ*, a freely available program developed by the National Institutes of Health (NIH) (http://rsbweb.nih.gov/ij/).

Briefly, each plate is scanned and then analysed using the CM Engine which detects each colony, and assigns each a numerical value based upon its circularity and size. The file output from the CM engine is then input into the DR engine; a web-based application that normalises raw screen data, and provides the user with an opportunity to manually select data for exclusion from analysis. The normalised data is used to calculate *p*-values by t-test for parametric data or the Mann-Whitney test for non-parametric data. The analysed data is annotated with colony identities and other information from a user-selected key file, and presented to the user in a tab-delimited downloadable text file.

Each tab-delimited text file can now be downloaded into the SV Engine, a web-based program on the Rothstein website (http://www.rothsteinlab.com/tools/) which presents the statistical data in a cartoon format for the user to compare the control and experimental plates. The program highlights colonies of interest based upon a user chosen p value, which by default is $p < 0.05$. The user selects the colonies of interest and the information surrounding the selected strains may then be downloaded to Microsoft Excel for further manipulation.

2.7 Relationship mapping of selected mutants

The ORFs selected as significant through ScreenMill analysis were imported into GeneMania (http://www.genemania.org/), and the Saccharomyces Genome Database Analysis tools "Gene Ontology Slim Mapper" (http://www.yeastgenome.org) to search for relationships between them. Relationships were parsed based upon products of the selected ORFs being part of a particular cellular component (e.g. Golgi apparatus), taking part in a particular biological process (e.g. RNA biosynthesis), or serving a specific molecular function (e.g. Transporter).

Chapter 3 Results

3.1 Screening the natural products derivative library against *F. graminearum*

The chemical library NDL-3000 (Natural Products Derivative) is composed of 3000 synthetic and synthetically modified pure natural compounds including but not limited to: alkaloids, phenolics, nucleoside analogs, carbohydrates, purines, pyrimidines, flavonoids, steroidal compounds, and natural amino acids. In addition a collection of plant extracts, provided by Andrew Waye of the University of Ottawa, and Antofine, a compound previously isolated from *Vincetoxicum rossicum* were used in this screen (Mogg *et al*, 2008).

A total of 538 of the 3000 compounds comprising the NDL-3000 collection, and 23 plant extracts were tested in liquid culture for inhibitory properties against *F. graminearum*. Briefly, *F. graminearum* spores expressing GFP were incubated at 28°C in rich medium amended with the different chemical compounds. Growth measurements were taken every 20 minutes over the course of 72 hours; an example of potential results is shown in Figure 2. To compare the relative growth between the treatments and the controls, the area beneath the curve was calculated and assigned a normalised growth value (NGV). Eight library compounds and three plant extracts (Table 1) were assigned an NGV < 0.1 ($p<0.05$) suggesting strong inhibitory properties, (red diamonds); twenty library compounds and twelve plant extracts (Table 2) were assigned an NGV value between 0.1 and 0.75 ($p<0.05$), suggesting growth suppressive properties (yellow circles); and five of the library compounds (Table 3) were assigned an NGV value greater than 1.75 ($p<0.05$) suggestive of growth enhancement (green squares).

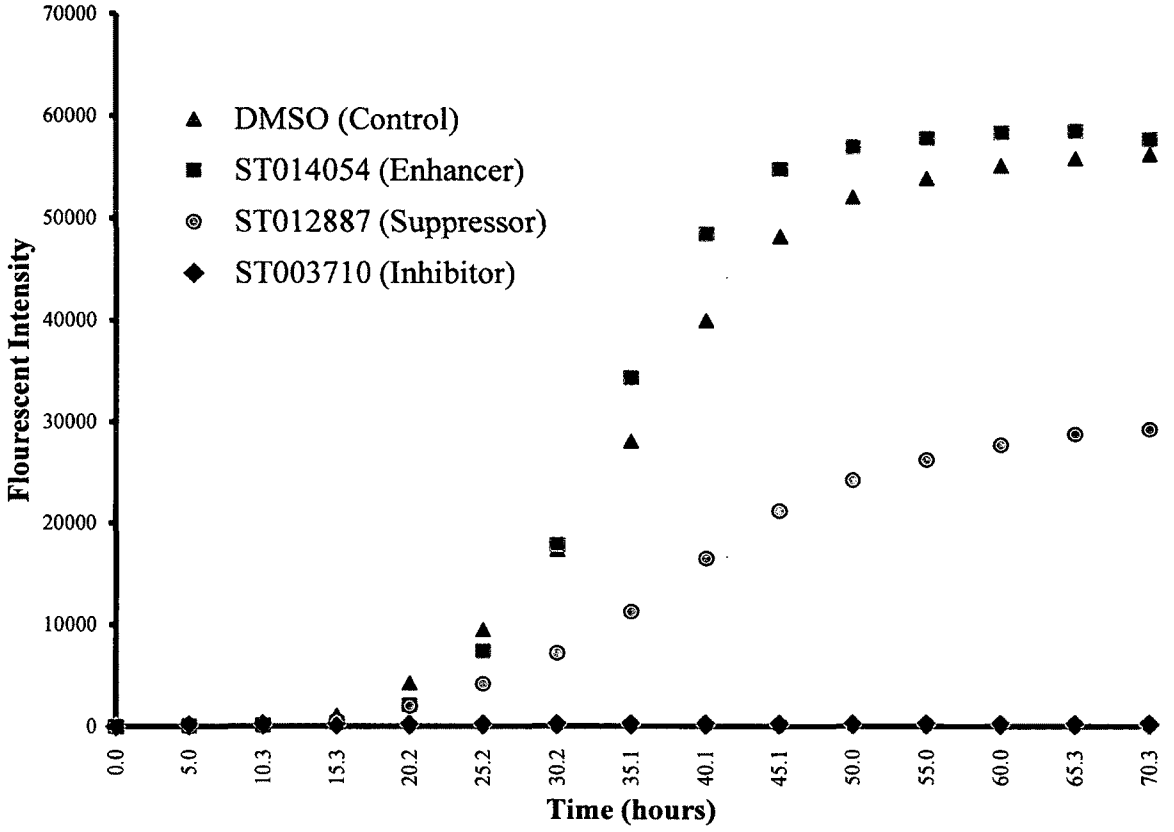

Figure 2: Sample results of NDL-3000 library screen against *F. graminearum* ZTE-2A. Conidia were grown in GYEP media amended with 0.07 µg/mL test compound, and incubated at 28°C for 72 hours; measurements at 520 nm were collected every 23 minutes. Each data point reflects a sampling period of 5 hour intervals.

Table 1: Compounds and plant extracts that inhibited *F. graminearum* growth

Compound ID [a]	Concentration [b]	Extraction Solvent [c]	Normalised Growth Value [d]	Coefficient of Variation [e]	*p*-value [f]
ST003709	0.07 mg/mL	~	0.016	0.03	1.84×10^{-3}
ST003710			0.017	0.05	1.85×10^{-3}
ST003712			0.001	0.05	1.05×10^{-5}
ST009866			0.011	0.57	8.70×10^{-3}
ST012830			0.005	0.21	4.52×10^{-8}
ST012844			0.022	0.07	4.90×10^{-8}
ST012947			0.008	0.30	4.53×10^{-6}
ST013063			0.011	0.30	2.51×10^{-8}
Antofine	0.150 mg/mL	EtOAc	0.003	0.35	8.25×10^{-6}
Chitosan	2 mg/mL	-	0.025	0.17	5.72×10^{-6}
White Pine - Wood (*Pinus strobus*)	1mg/mL	EtOAc	0.059	0.34	1.28×10^{-5}

[a] Compound identification number from TimTec Library, or source of crude plant extract.

[b] Final concentration in GYEP media at which compound was tested against *F. graminearum*

[c] Solvent used to produce the crude plant extracts

[d] Normalised Growth value was calculated as NGV = [average AUC_{Test}]/[average $AUC_{Control}$], where [average AUC_{Test}] = average area under the curve of the experimental growth plot between 0-72 hours, [average $AUC_{Control}$] = average area under the curve of the control growth plot between 0-72 hours.

[e] Coefficient of Variation was calculated as CV = [Standard Deviation of replicates]/[Mean of Replicates] (raw data not shown)

[f] Two-tailed t-test with an assumed equal variance comparing the experimental AUC against the control AUC.

Table 2: Compounds and plant extracts that suppressed *F. graminearum* growth

Compound ID [a]	Concentration [b]	Extraction Solvent [c]	Normalised Growth Value [d]	Coefficient of Variation [e]	*p*-value [e]
ST001473	0.07 mg/mL	~	0.317	0.09	3.7×10^{-3}
ST002041			0.654	0.10	2.8×10^{-3}
ST002052			0.731	0.06	3.3×10^{-3}
ST003711			0.576	0.05	2.85×10^{-5}
ST004333			0.695	0.12	3.2×10^{-3}
ST004334			0.607	0.18	3.8×10^{-3}
ST004335			0.675	0.11	2.0×10^{-3}
ST005073			0.558	0.29	1.4×10^{-2}
ST007461			0.689	0.03	1.0×10^{-4}
ST009315			0.726	0.07	3.3×10^{-3}
ST009843			0.440	0.05	1.0×10^{-4}
ST010212			0.687	0.03	2.0×10^{-4}
ST011608			0.747	0.09	4.9×10^{-3}
ST011722			0.722	0.08	2.2×10^{-3}
ST012878			0.581	0.04	1.35×10^{-5}
ST012884			0.347	0.001	4.23×10^{-4}
ST012887			0.550	0.01	1.29×10^{-3}
ST012921			0.129	0.14	9.63×10^{-6}
ST012954			0.399	0.09	1.21×10^{-5}
ST014829			0.458	0.04	5.0×10^{-4}
Balsam Fir - Wood (*Abies balsamea*)	1 mg/mL	MeOH	0.382	0.11	1.1×10^{-3}
Black Spruce - Bark (*Picea mariana*)	2 mg/mL	EthOH	0.277	0.06	7.24×10^{-7}
E. Hemlock - Wood (*Tsuga canadensis*)	1 mg/mL	MeOH	0.537	0.12	7.90×10^{-4}
E. Hemlock - Wood (*Tsuga canadensis*)	1 mg/mL	EtOAc	0.716	0.15	1.4×10^{-2}
Jack Pine - Bark (*Pinus banksiana*)	2 mg/mL	EthOH	0.239	0.01	1.98×10^{-7}
Juniper - Leaf (*Juniperus communis*)	1 mg/mL	EthOH	0.345	0.04	2.53×10^{-5}
Juniper - Leaf (*Juniperus communis*)	1 mg/mL	MeOH	0.449	0.06	6.75×10^{-5}
Juniper - Wood (*Juniperus communis*)	1 mg/mL	MeOH	0.134	0.16	1.01×10^{-5}
Juniper - Wood (*Juniperus communis*)	1 mg/mL	EthOH	0.140	0.10	1.84×10^{-4}
Tamarack - Wood (*Larix laricina*)	1 mg/mL	MeOH	0.591	0.10	8.2×10^{-4}
White Cedar - Wood (*Thuja occidentalis*)	1 mg/mL	MeOH	0.433	0.05	6.14×10^{-5}
White Spruce - Bark (*Picea glauca*)	2 mg/mL	EthOH	0.310	0.10	4.67×10^{-6}

[a] Compound identification number from TimTec Library, or source of crude plant extract.
[b] Final concentration in GYEP media at which compound was tested against *F. graminearum*
[c] Solvents used to produce the crude plant extracts
[d] Normalised Growth Value was calculated as NGV= [average AUC_{Test}]/[average $AUC_{Control}$],
[e] Coefficient of Variation was calculated as CV = [Standard Deviation of replicates]/[Mean of Replicates] (raw data not shown)
[f] Two-tailed t-test with an assumed equal variance comparing the experimental AUC against the control AUC.

Table 3: Compounds that enhanced growth properties of *F. graminearum*

Compound [a]	Concentration [b]	Normalised Growth Value [c]	Coefficient of Variation [d]	*p*-value [e]
ST001352	0.07 mg/mL	1.765	0.05	3.3×10^{-3}
ST009474		3.398	0.14	1.0×10^{-3}
ST009870		1.896	0.06	2.82×10^{-3}
ST014054		1.919	0.07	4.0×10^{-4}
ST014140		1.765	0.07	8.0×10^{-4}

[a] Compound identification number from TimTec Library.

[b] Final concentration in GYEP media at which compound was tested against *F. graminearum*

[c] Normalised Growth value was calculated as NGV = [average AUC_{Test}]/[average $AUC_{Control}$], where [average AUC_{Test}] = average area under the curve of the experimental growth plot between 0-72 hours, [average $AUC_{Control}$] = average area under the curve of the control growth plot between 0-72 hours.

[d] Coefficient of Variation was calculated as CV = [Standard Deviation of replicates]/[Mean of Replicates] (raw data not shown)

[e] Two-tailed t-test with an assumed equal variance comparing the experimental AUC against the control AUC.

3.1.1 Verification of inhibitory properties of compounds against *F. graminearum*

Twenty-one compounds from the initial screen, Antofine, and 15 additional chemicals from the Timtec library, were selected for further investigation (Table 4). The 15 additional compounds were chosen based upon their structural similarity to the original 21 selected. To confirm the observations in the initial screen, and to establish a working concentration to be used in the next stage of the investigation, two sets of serial dilution experiments were undertaken. A serial dilution from 0.1 mg/mL to 0.0125 mg/mL (two-fold) or 0.1 mg/mL to 0.0001 mg/mL (ten-fold) were tested under the same growth and measurement conditions as the initial screen. The compounds were rated for efficacy based upon the criteria outlined in section 3.1. Each treatment was assigned an NGV value based upon the method outlined in section 2.3.1.

Twenty eight of the 37 compounds screened demonstrated similar effects upon *F. graminearum* growth as observed during the initial screen. Nineteen of the 22 compounds originally selected as inhibitory were confirmed, while six more were identified from the 15 additional compounds selected for chemical similarity (Table 4). The compounds were categorized into seven groups, based upon structural similarity. Since the majority of these compounds do not have common names, each chemical is referred to by the number assigned to it within the TimTec library (http://www.timtec.net).

Table 4: Comparison of original and verification screen on *F. graminearum* growth

Chemical ID [a]	Original Screen [b]	Verification Screen [c]	Chemical ID [a]	Original Screen [b]	Verification Screen [c]
ST001473	Suppressor	Same	**ST007461**	Suppressor	Same
ST002032	No effect	Same	**ST007497**	No effect	Same
ST002037	No effect	Same	**ST009866**	Inhibitor	Same
ST002041	Suppressor	No effect	**ST009867**	No effect	Same
ST002051	No effect	Same	**ST009870**	Enhancer	No effect
ST002052	No effect	Same	**ST009871**	No effect	Same
ST003704	No effect	Suppressor	**ST012842**	No effect	Suppressor
ST003705	No effect	Suppressor	**ST012844**	Inhibitor	Same
ST003707	No effect	Suppressor	**ST012878**	Suppressor	Same
ST003709	Inhibitor	Same	**ST012879**	Suppressor	Same
ST003710	Inhibitor	Same	**ST012884**	Suppressor	Same
ST003711	Suppressor	Same	**ST012887**	Suppressor	Same
ST003713	No effect	Suppressor	**ST012896**	Suppressor	Same
ST003714	No effect	Suppressor	**ST012945**	No effect	Same
ST004333	Suppressor	Same	**ST012946**	No effect	Same
ST004334	Suppressor	Same	**ST012947**	Inhibitor	Same
ST004335	Suppressor	Same	**ST013063**	Inhibitor	Same
ST005130	Suppressor	Same	**ST014054**	Enhancer	No effect
ST005134	Suppressor	Same			

[a] Compound identification number from TimTec Library.

[b] The results of the original library screen where "inhibitor" reduced growth to less than 10% of that observed in the control, "suppressor" reduced growth to between 10% and 75% of that observed in the control, "No effect" exhibited a growth rate within 25% of that observed in the control, and "enhancer" which demonstrated a growth rate 175% or greater of that observed in the control.

[c] The results of the verification screen use the same annotations as "b", but also includes "same" meaning that the conclusions reached in the verification screen are identical to those reached in the initial library screen.

The members of group 1 (Figure 3) were assembled based upon the common benizimidazole back-bone structure (highlighted by the brackets in Figure 3), a known class of fungicide the primary mode of action of which is believed to be the disruption of microtubule assembly which impacts upon cell division, cell motility, cellular secretion, nutrient absorption and intercellular transport (Danaher *et al*, 2007). All of the members also had one side chain (menthol) in common (R1) with a variation in their second side chain (R2) and/or a change in the location of the double bond in the five member ring, (denoted by the star). The compounds were tested by two-fold serial dilution at concentrations of 0.1 mg/mL to 0.0125 mg/mL (ST003704, ST003705, ST003707, ST003711, ST003713, ST003714), or by ten-fold serial dilution from 0.1 mg/mL to 0.0001 mg/mL (ST003709 and ST003710). Five of the group 1 compounds required a concentration of 0.1 mg/mL to reduce the growth of *F. graminearum* to less than 25% of that observed in the uninhibited control (Table 5). The remaining compounds proved to be efficacious at lower concentrations (Table 5), however, there did not appear to be any obvious trend in side chain composition to account for increased efficacy. At concentrations less than those listed, the calculated NGV value was greater than 0.81 in all cases (data not shown).

Table 5: Chemicals in group 1 suppress *F. graminearum* growth

Chemical ID [a]	Concentration [b]	Normalised Growth Value [c]	Coefficient of Variation [d]	*p*-value [e]
ST003711	0.1 mg/mL	0.001	0.09	3.36×10^{-5}
ST003709	0.1 mg/mL	0.001	0.03	2.14×10^{-4}
ST003710	0.1 mg/mL	0.002	0.16	9.69×10^{-6}
ST003705	0.1 mg/mL	0.031	0.37	4.01×10^{-5}
ST003704	0.1 mg/mL	0.003	0.20	3.38×10^{-5}
ST003714	0.05 mg/mL	0.004	0.19	2.99×10^{-8}
ST003713	0.025 mg/mL	0.001	0.03	1.28×10^{-9}
ST003707	0.0125 mg/mL	0.32	0.05	1.24×10^{-5}

[a] Compound identification number from TimTec Library.

[b] The lowest compound concentration in GYEP at which the average normalised value was 0.75 or less.[c] Normalised Growth Value was calculated as NGV = [average AUC_{Test}]/[average $AUC_{Control}$], where [average AUC_{Test}] = average area under the curve of the experimental growth plot between 0-72 hours, [average $AUC_{Control}$] = average area under the curve of the control growth plot between 0-72 hours.

[d] Coefficient of Variation was calculated as CV = [Standard Deviation of replicates]/[Mean of Replicates] (raw data not shown)

[e] Two-tailed t-test with an assumed equal variance.

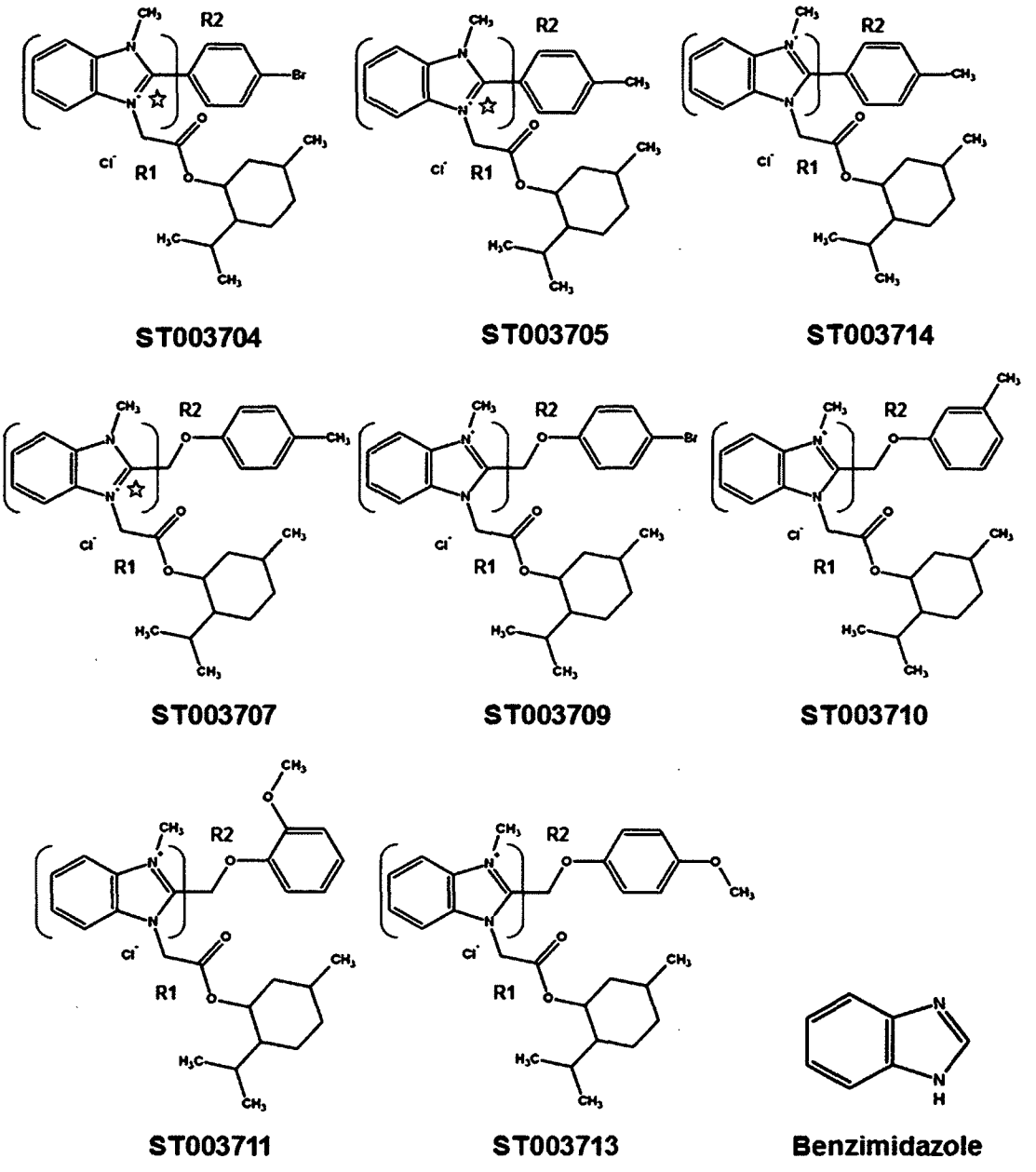

Figure 3: Chemical compounds in group 1

Common benzimidazole backbone is emphasized in brackets, a change in double bond location is denoted by the star.

The members of group 2 were assembled based upon the bracketed common structure "gossypol", of which all of these compounds are derived (Figure 4). Gossypol is a polyphenolic compound first derived from the cotton plant (Genus *Gossypium*) that has numerous biological activities attributed to it including, contraceptive, antiviral, anticancer, and antimicrobial properties (Wang *et al*, 2009). The members of group 2 were tested by two-fold serial dilution at concentrations of 0.1 mg/mL to 0.0125 mg/mL and all treatments demonstrated a 25% or greater reduction in growth (NGV < 0.75) at a concentration of 0.1 mg/mL or less in comparison with the untreated control. Seven of the 10 compounds suppressed *F. graminearum* growth to below 55% of that observed in the uninhibited control at a concentration of 0.0125 mg/mL or greater (Table 6). The remaining compounds proved efficacious at concentrations at or less than 0.1 mg/mL (Table 6) and I did not observe any obvious trend in side chain composition to account for the differences in efficacy.

Table 6: Chemicals in group 2 suppress *F. graminearum* growth

Chemical ID [a]	**Concentration** [b]	**Normalised Growth Value** [c]	**Coefficient of Variation** [d]	***p*-value** [e]
ST005134	0.1 mg/mL	0.48	0.09	2.92×10^{-5}
ST005130	0.05 mg/mL	0.46	0.05	4.59×10^{-6}
ST012887	0.05 mg/mL	0.47	0.11	1.46×10^{-4}
ST012879	0.0125 mg/mL	0.25	0.06	5.52×10^{-7}
ST012896	0.0125 mg/mL	0.29	0.10	3.43×10^{-6}
ST012878	0.0125 mg/mL	0.36	0.07	3.87×10^{-6}
ST004335	0.0125 mg/mL	0.40	0.02	7.75×10^{-8}
ST004334	0.0125 mg/mL	0.52	0.03	1.89×10^{-6}
ST012884	0.0125 mg/mL	0.55	0.07	4.58×10^{-5}
ST004333	0.0125 mg/mL	0.55	0.05	8.99×10^{-6}

[a] Compound identification number from TimTec Library.

[b] The lowest compound concentration in GYEP at which the average normalised value was 0.75 or less.

[c] Normalised Growth Value (NGV) = [average AUC_{Test}]/[average $AUC_{Control}$], where: [average AUC_{Test}] = average area under the curve of the experimental growth plot between 0-72 hours, [average $AUC_{Control}$] = average area under the curve of the control growth plot between 0-72 hours.

[d] Coefficient of Variation was calculated as CV = [Standard Deviation of replicates]/[Mean of Replicates] (raw data not shown)

[e] Two-tailed t-test with an assumed equal variance.

The members of group 2 were unique among the seven groups tested in that the rate of inhibition was proportional to the concentration of the compound. For example, the NGV values for treatment with ST012896 approximately double as the concentration of the compound was halved (Table 7). *F. graminearum* spores treated with the other compound groups displayed a moderate reduction in growth until a certain critical concentration was reached, at which point the growth was usually strongly suppressed, suggesting a threshold limit.

Table 7: Concentration of ST012896 is proportional to inhibition

Concentration [a]	**Normalised Growth Value** [b]	**Coefficient of Variation** [c]	***p*-value** [d]
0.1 mg/mL	0.036	0.06	1.25×10^{-7}
0.05 mg/mL	0.093	0.02	1.59×10^{-6}
0.025 mg/mL	0.182	0.04	9.48×10^{-8}
0.0125 mg/mL	0.290	0.10	1.59×10^{-6}

[a] Concentration of compound ST012896 in GYEP.
[b] Normalised Growth Value = [average AUC_{Test}]/[average $AUC_{Control}$], where [average AUC_{Test}] = average area under the curve of the experimental growth plot from 0-72 hours, [average $AUC_{Control}$] = average area under the curve of the control growth plot from 0-72 hours.
[c] Coefficient of Variation (CV) = [Standard Deviation of replicates]/[Mean of Replicates] (raw data not shown)
[d] Two-tailed t-test with an assumed equal variance.

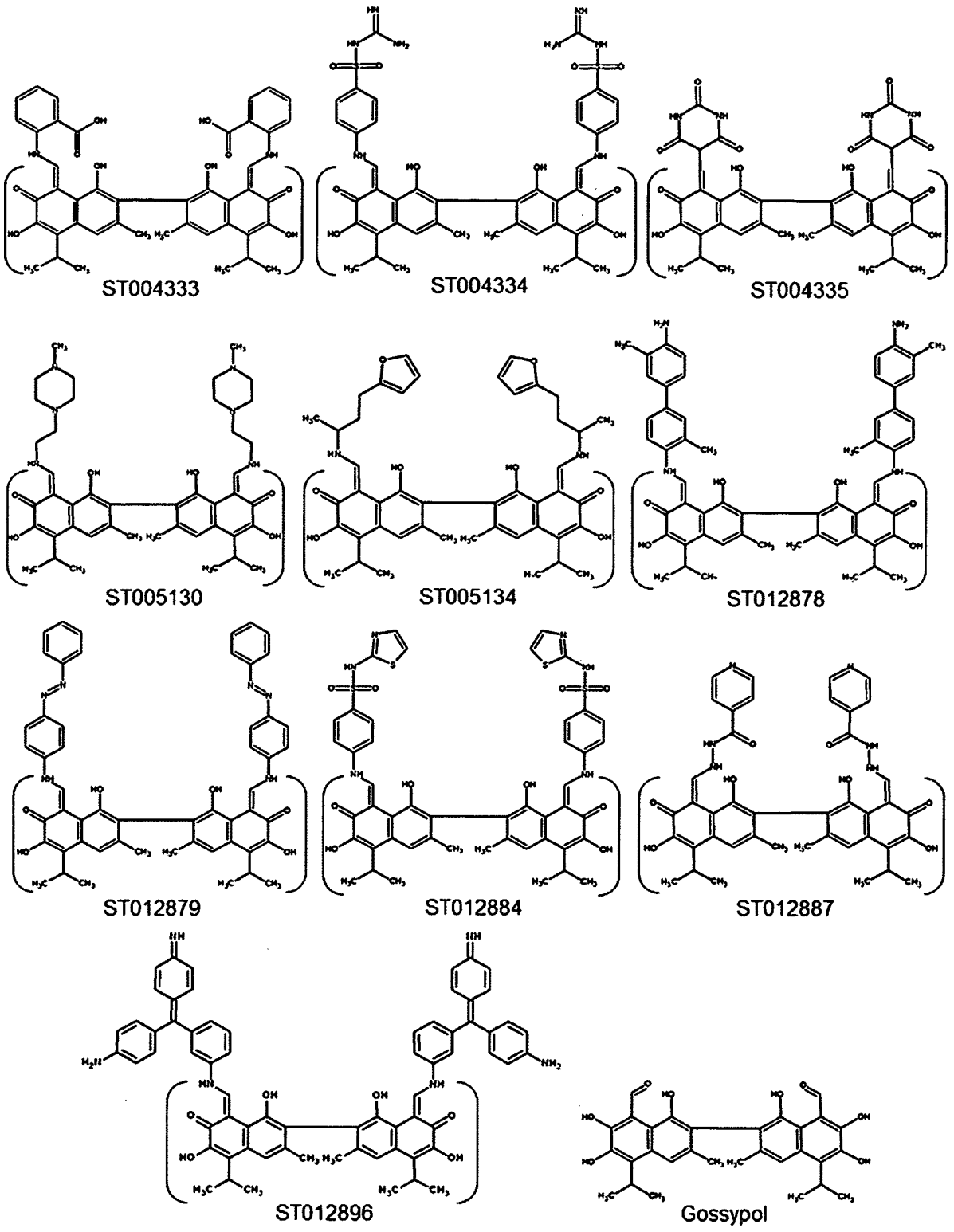

Figure 4: Chemical compound in group 2
Common gossypol backbone is emphasized in brackets

The members of group 3 were categorised based upon the bracketed common carboxylic acid structure (Figure 5). The members of group 3 were tested against *F. graminearum* by two-fold serial dilution at concentrations of 0.1 mg/mL to 0.0125 mg/mL. Compound ST007497 did not appreciably reduce the rate of growth of *F. graminearum* even at the highest concentration tested (0.1 mg/mL) (NGV = 0.9355, CV = 0.04, p = 0.03713). The compound ST007461 inhibited growth at 0.1 mg/mL (NGV = 0.0352, CV = 0.60, $p = 2.15 \times 10^{-7}$), consistent with what was observed during the original screen. A normalised value of 0.80 or greater was calculated for both compounds at concentrations less than 0.1 mg/mL (data not shown). It is not obvious whether it is the identity of the halogen, or the orientation and attachment position of the indole group side chain that is responsible for the difference in efficacy.

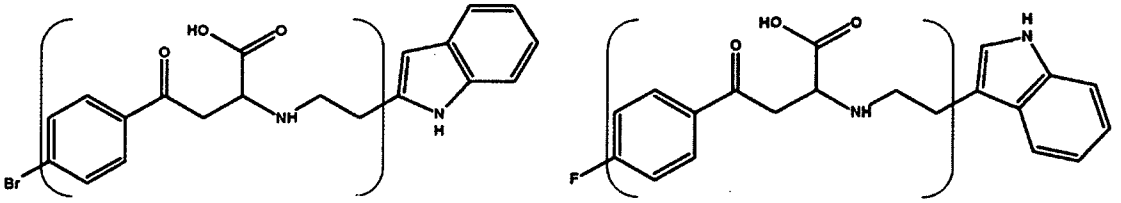

Figure 5: Chemical compounds in group 3
Common backbone is emphasized in brackets

The three members of group 4 (Figure 6) were assembled based upon the bracketed common structure (Figure 6). The members of group 4 were tested by two-fold serial dilution at concentrations of 0.1 mg/mL to 0.0125 mg/mL. Spores treated with ST014054 at 0.1 mg/mL (NGV = 0.8502,CV = 0.11, $p = 0.0513$) or compound ST009867 at 0.1 mg/mL (NGV = 0.9187, CV = 0.03, $p = 0.0154$) did not demonstrate a significant reduction in growth sufficient to satisfy the selection criteria. Treatment with ST009866 however, resulted in suppression of growth at concentrations 0.0125 mg/mL (NGV = 0.3129, CV = 0.10, $p = 1.41 \times 10^{-5}$) or greater.

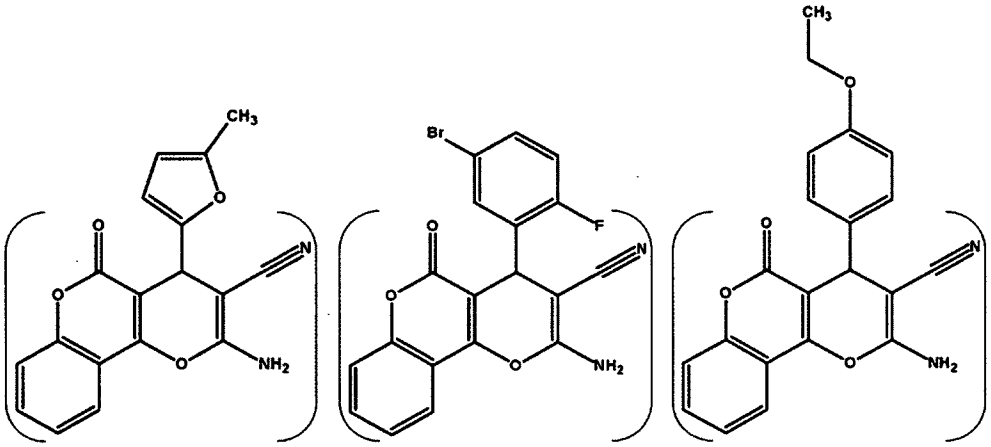

Figure 6: Chemical compounds in group 4
Common backbone is emphasized in brackets

Group five (Figure 7), composed of compounds ST012842 and ST012844 have an identical backbone, highlighted by the brackets. Both compounds were tested using ten-fold serial dilution from 0.1 mg/mL to 0.0001 mg/mL and two-fold serial dilution from 0.1 mg/mL to 0.0125 mg/mL. Compound ST012842 demonstrated growth suppression at 0.05 mg/mL (NGV = 0.736, CV = 0.07, $p = 0.0012$) and inhibition at 0.1 mg/mL (NGV = 0.076, CV = 0.40 $p = 2.82 \times 10^{-5}$). In contrast compound ST012844 demonstrated inhibition at concentrations as low as 0.01 mg/mL (NGV = 0.0125, CV = 0.35, $p = 2.94 \times 10^{-7}$). The side chain is the only variation between the two molecules, with an acridine group attached to ST012842, and a phenazone group attached to ST012844.

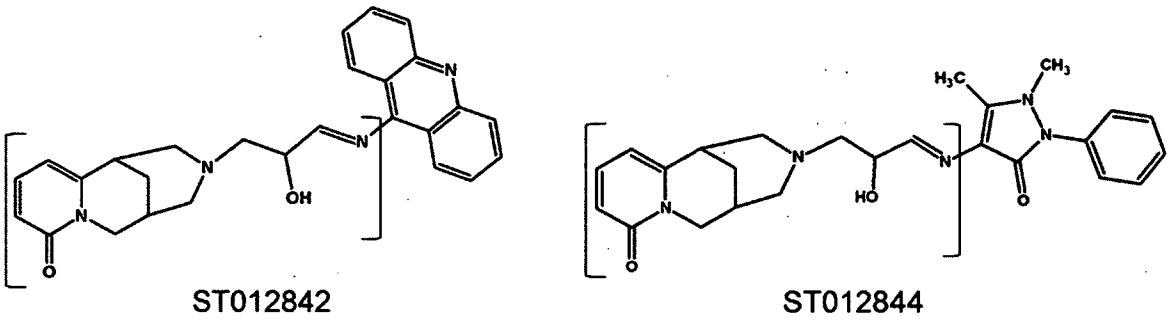

Figure 7: Chemical compounds in group 5
Common backbone is emphasized in brackets

Compound group 6 (Figure 8) are derivatives of ST012946 [(2E)-3-phenyl-1-(2-(3-pyridyl)piperidyl)prop-2-en-1-one], denoted by the brackets. Compounds were all tested using ten-fold serial dilutions from 0.1 mg/mL to 0.0001 mg/mL. Treatment with compound ST012945 did not demonstrate a reduction in growth of sufficient significance

to satisfy the selection criteria even at the highest concentration tested, 0.1 mg/mL (NGV = 0.9042, CV = 0.02, $p = 0.0019$). The same observation was recorded for treatment with compound ST012946 (NGV = 0.9375, CV = 0.07, $p = 0.0702$). Treatments using the last compound ST012947 demonstrated complete inhibition at 0.1 mg/mL (NGV = 0.0007, CV = 0.15, $p = 3.81 \times 10^{-9}$), but no significant effect at 0.01 mg/mL (NGV = 0.9450, $p = 0.0268$). A normalised value greater than 0.90 was calculated for all compounds at all other concentrations tested (data not shown).

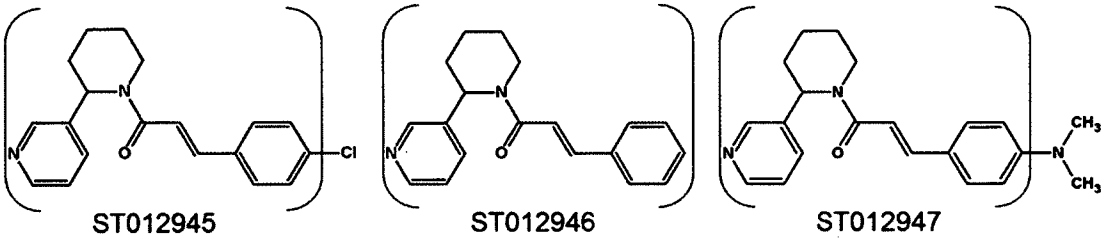

Figure 8: Chemical compounds in group 6
Common backbone is emphasized in brackets

Finally, the three compounds with no similarities to other groups were tested by serial dilution at concentrations of 0.1 mg/mL to 0.0125 mg/mL (ST001473) and 0.1 mg/mL to 0.0001 mg/mL (ST013063). Treatments using compound ST001473 demonstrated growth suppression at 0.1 mg/mL (NGV = 0.6370, CV = 0.03, $p = 0.0001$), and no significant effect upon growth at lower concentrations. Treatments with compound ST013063 demonstrated complete inhibition at 0.1 mg/mL (NGV = 0.0074, CV = 0.04, $p = 3.92 \times 10^{-9}$), growth suppression at 0.01 mg/mL (NGV = 0.7635, CV = 0.04, $p = 5.56 \times 10^{-4}$), no significant reduction in growth at concentrations of 0.001 mg/mL or less. Compound ST001473 has the common name 4-chlorochalcone, classifying it as a chalcone derivative, a class of natural products that have antifungal

properties associated with them (Lahtchev *et al*, 2008). The compound Antofine has previously been demonstrated to be inhibitory against other strains of *F. graminearum* (NRRL K062 and CBS 415.86) at concentrations ranging from 3 μg/mL to 6 μg/mL, and *F. oxysporum* (JB1D411) at 4 μg/mL (Mogg *et al*, 2008).

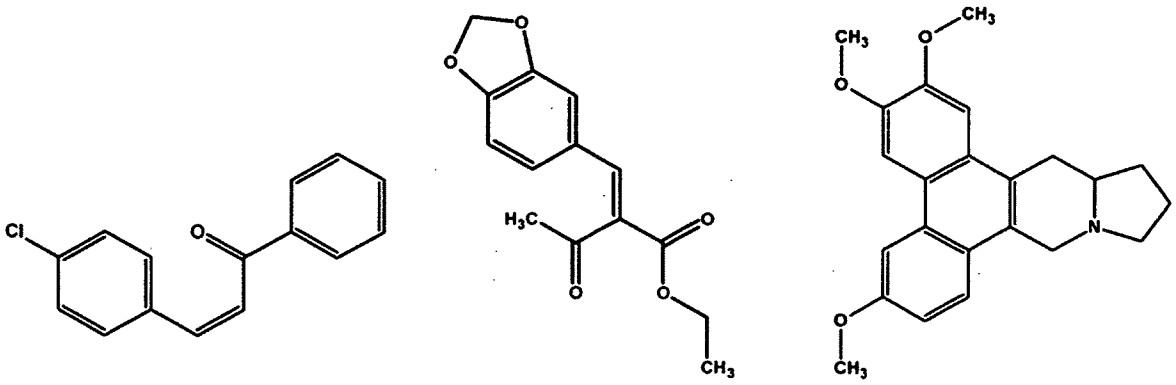

Figure 9: Chemical compounds in group 7

In summary, the second verification screen validated the majority of the results observed during the initial screen. The majority of the compounds selected for verification screening contained nitrogen, present as examples of primary, secondary, tertiary and aromatic amines. Of the 37 compounds screened, 28 behaved as previously observed; six additional growth perturbing compounds were identified from the remaining nine. Three of the seven groups identified contain or are derivatives of, compounds which have known biological activities, demonstrating that this method of seeking antifungal compounds is effective.

The majority of the compounds, with the exception of those belonging to group 2, exhibited critical dosage on *F. graminearum* growth; that is growth perturbation was moderate until a particular threshold dosage was passed, at which point growth inhibition became severe. In contrast, the compounds of group 2 demonstrated a linear relationship between growth suppression and compound concentrations; that is, the greater the concentration of inhibitor, the greater the reduction in growth that was observed, without complete inhibition of all growth even at the highest concentrations tested.

Based upon the results of the verification screen, four compounds ST003707, ST004335, ST009866 and ST012844 from the TimTec library and the plant isolate Antofine were selected for further studies.

3.2 Chemical perturbation of genetic interactions in *S. cerevisiae*

Prior to testing the compounds against the *S. cerevisiae* haploid deletion mutant library, the compounds were first assessed against the wild type *S. cerevisiae* BY4741 (Haploid) and BY4743 (diploid) in liquid culture using YPD broth. The rate of growth of the organisms was monitored using a method similar to that used to assess the growth of *F. graminearum*. For this assay the chosen value of significance was the half maximal inhibitory concentration (IC_{50}), which is defined as the concentration of inhibitor required to reduce the rate of growth of a culture by half, relative to an uninhibited culture of the same type. The results are summarised in Table 8.

Table 8: IC_{50} for *S. cerevisiae* BY4741 (Haploid) and BY4743 (diploid)

Compound ID[a]	Strain [b]	IC_{50} Concentration Range [c]	Normalised Growth Value [d]	Coefficient of Variance [e]	*p*-value [f]
ST003707	BY4741	0.02 - 0.0225 mg/mL	0.478	0.13	7.26×10^{-5}
	BY4743	0.0175 - 0.02 –mg/mL	0.152	0.23	2.74×10^{-4}
ST004335	BY4741	0.4 - 0.8 mg/mL	0.329	0.12	8.13×10^{-6}
	BY4743	0.4 - 0.8 mg/mL	0.262	0.15	4.41×10^{-6}
ST009866	BY4741	< 0.06 mg/mL	0.401	0.06	4.74×10^{-6}
	BY4743	0.08 - 0.09 mg/mL	0.437	0.06	6.09×10^{-6}
ST0012844	BY4741	0.16 - 0.32 mg/mL	0.009	0.10	2.79×10^{-7}
	BY4743	0.08 - 0.16 mg/mL	0.448	0.04	3.40×10^{-6}
Antofine	BY4741	1.1-1.4 µg/mL	0.402	0.12	4.43×10^{-5}
	BY4743*	0.47 µg/mL*	~	~	~

[a] Compound identification number from TimTec Library.
[b] Strain of *S. cerevisiae* tested in YPD_{BROTH}, where BY4741 is haploid, and BY4743 is diploid.
[c] Compound concentration range between which the IC_{50} value would be located.
[d] Normalised Growth Value (NGV) = [average AUC_{Test}]/[average $AUC_{Control}$], where [average AUC_{Test}] = average area under the curve of the experimental growth plot between 0-48 hours, [average $AUC_{Control}$] = average area under the curve of the control growth plot between 0-48 hours. The value reported is associated with the greater concentration of the range.
[e] Coefficient of Variation was calculated as CV = [Standard Deviation of replicates]/[Mean of Replicates] (raw data not shown)
[f] Two-tailed t-test with an assumed equal variance.
* Reported IC_{50} for *S. cerevisiae* BY4743 was not measured by plate reader, but was assessed by visual inspection. Consequently a NGV or *p*-value cannot be calculated.

Using the concentrations listed in Table 8 as a reference point, the compounds were tested against *S. cerevisiae* BY4741 (haploid) and BY4743 (diploid) in YPD solid agar plates. This was necessary since the yeast deletion library was to be screened on solid agar plates.

With the exception of Antofine, ST009866 and ST012844, the concentration and quantities of the selected library compounds required to inhibit yeast growth in solid format were much greater than was necessary to inhibit the growth in liquid culture. For example, the compound ST003707 inhibited *S. cerevisiae* growth at concentrations greater than 0.025 mg/mL in solid (compared to 0.0225 mg/mL in liquid) and ST009866 inhibited *S. cerevisiae* growth at concentrations greater than 0.8 mg/mL in solid (compared to 0.06 mg/mL in liquid).

The quantities required to screen the complete haploid yeast library against any compound other than Antofine would consume all, or at least a significant portion of what was currently available, leaving very little margin for error or further experimentation. The decision was made to hold these compounds in reserve and concentrate instead upon Antofine, which was efficacious, and the method by which more could be isolated has already been established (Mogg *et al*, 2008).

3.3 Antofine modulates cellular responses in *S. cerevisiae*

In preparation for further work a new and fresh supply of Antofine was isolated. Briefly, fresh *V. rossicum* root tissue was harvested, vacuum desiccated, and extracted using ethyl acetate. Antofine was isolated from the crude extract through acid-base solvent extraction and portioning, followed by preparatory HPLC, and finally purified using Thin Layer Chromatography. Antofine was then subjected to ^{1}H NMR to verify identify and purity (Figure 10 A).

3.3.1 Proton Nuclear Magnetic Resonance spectroscopy of Antofine

Proton NMR is a method by which the molecular structure of a compound can be determined through the measurement of the resonance frequency of a hydrogen atom in an applied magnetic field (Pavia *et al*, 2001). The purified Antofine was dissolved in $CDCl_3$ and was subjected to ^{1}H NMR spectroscopy. As shown in Figure 10 B, the NMR spectra of Antofine purified from *Vincetoxicum rossicum* corresponded to that published by Kim *et al* (2003), confirming that the isolated compound was Antofine.

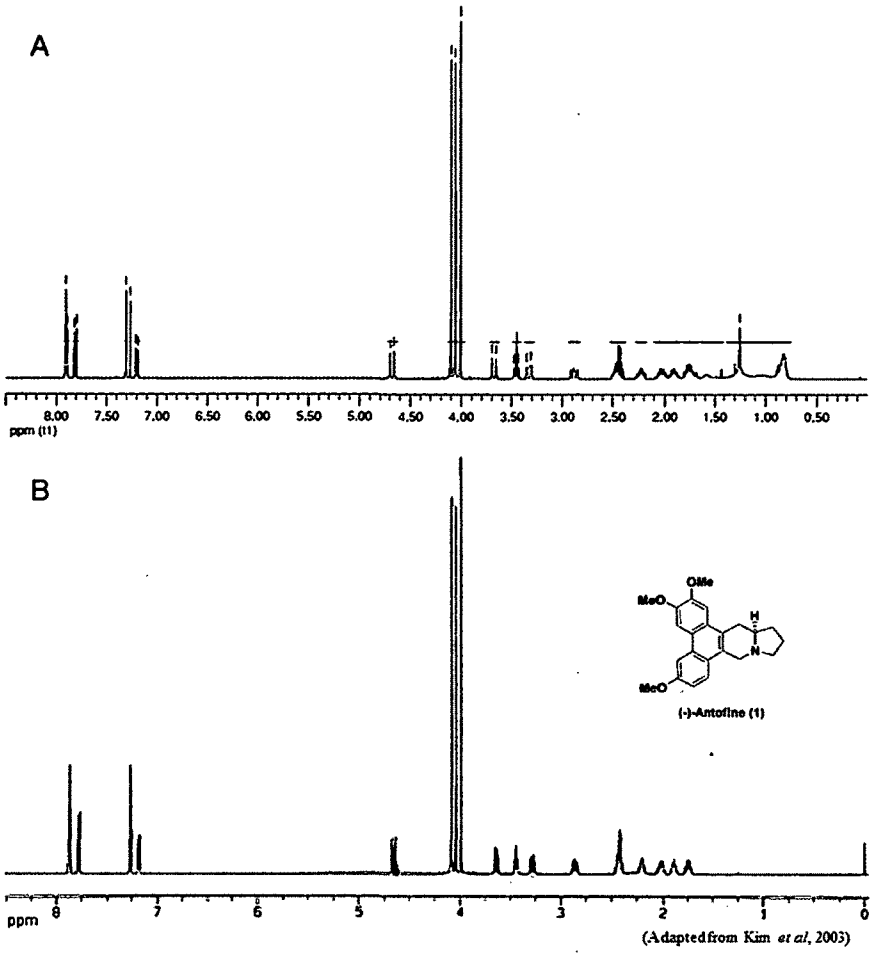

Figure 10: ^{1}H NMR of antofine

A) Antofine purified by TLC was subjected to proton NMR spectrocopy ($CDCl_3$, 400MHz): peaks rep by δ 7.90 (s, 1H), 7.89 (d, J=2.6Hz, 1H), 7.81 (d, J=9.1Hz, 1H), 7.30 (s, 1H), 7.20 (dd, J=9.0, 2.5Hz, 1H), 4.68 (d, J=14.9Hz, 1H), 4.10 (s, 3H), 4.06 (s, 3H), 4.01 (s, 3H), 3.68 (br d, J=15.0Hz, 1H), 3.45 (td, J=8.5, 1.9Hz, 1H), 3.33 (dd, J=15.8, 2.4Hz, 1H), 2.91-2.85 (m, 1H), 2.51-2.40 (m, 2H), 2.27-2.19 (m, 1H), 2.09-1.97 (m, 1H), 1.96-1.86 (m, 1H), 1.81-1.71 (m, 1H).

B) Accepted ^{1}H NMR spectrum published by Kim *et al* (2003); ^{1}H NMR (600 MHz, CDCl3) δ 1.71-1.78 (m, 1H), 1.86-1.92 (m, 1H), 1.98-2.04 (m, 1H), 2.18-2.23 (m,1H), 2.39-2.46 (m, 2H), 2.83-2.88 (m, 1H), 3.28 (dd, J = 15.6, 2.4 Hz, 1H), 3.44 (dt, J = 1.8, 8.5 Hz, 1H), 3.64 (d, J = 14.7 Hz, 1H), 3.99 (s, 3H), 4.04 (s, 3H), 4.08 (s, 3H), 4.66 (d, J = 14.7 Hz, 1H), 7.18 (dd, J = 9.0, 2.5 Hz, 1H), 7.27 (s, 1H), 7.78 (d, J = 9.0 Hz, 1H), 7.86 (d, J = 2.5 Hz, 1H), 7.87 (s, 1H).

3.3.2 Establishment of sub-lethal concentration of Antofine against *S. cerevisiae* in solid media

Before screening the haploid mutant library, the sub-lethal concentration required to suppress yeast growth in solid medium had to be established. Antofine was tested using the same method as that used to screen the library compounds in solid media (Section 2.5.1 and Section 3.2). A concentration of 0.20 µg/mL was selected as a sub-lethal concentration, based upon a reduction in growth of the yeast spots (Figure 11); the haploid yeasts displayed greater sensitivity to Antofine than the diploid strains.

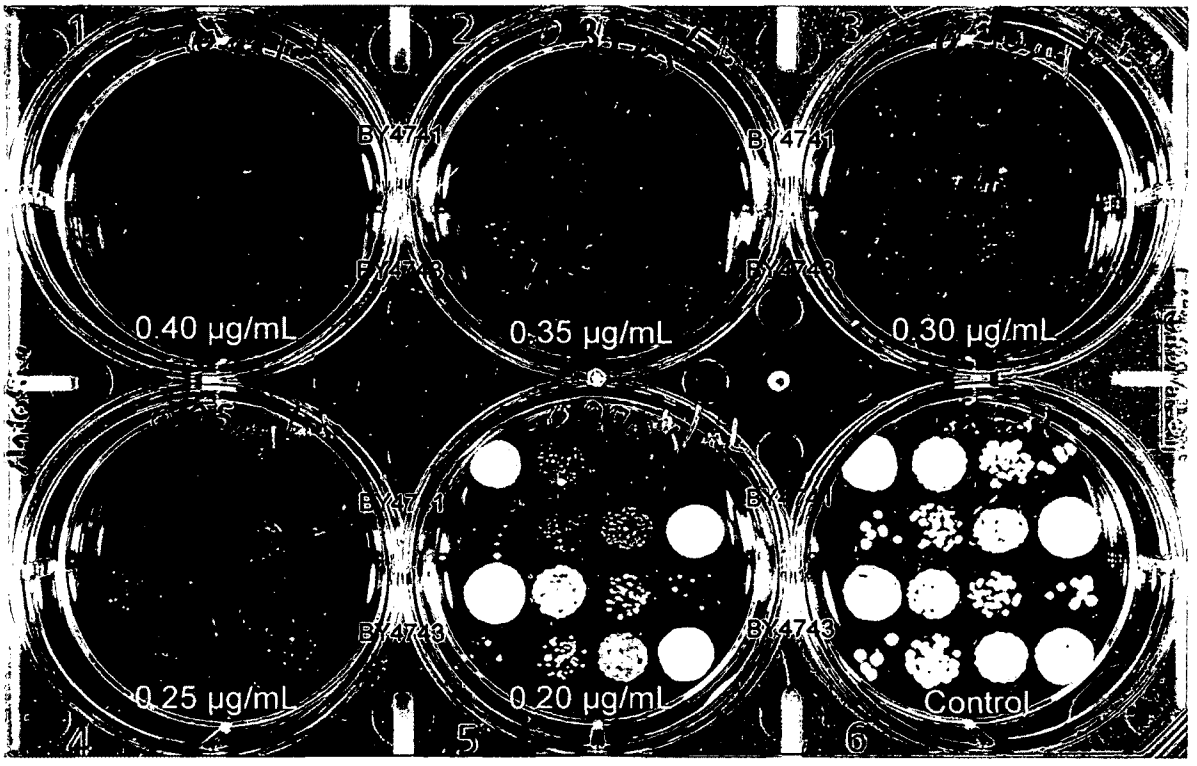

Figure 11: Antofine inhibition of *S. cerevisiae* growth in solid media.
Two strains of *S. cerevisiae* BY4741 (haploid) and BY4743 (diploid) spotted in ten-fold dilution onto 1.5% YPD_{AGAR} into which Antofine has been dissolved. Each well contains 2 mL of 1.5% YPD_{AGAR} + Antofine, diluted with final concentrations (Left to right, top to bottom) 0.40 µg/mL, 0.35 µg/mL, 0.30 µg/mL, 0.25 µg/mL, 0.20 µg/mL, and Control. The upper two rows of yeast are BY4741 (haploid), and the lower two rows are BY4743 (diploid). The yeasts have been spotted as a 2 µL aliquot, arranged counter to each other. Stock yeast cultures are set at 1.5 x 10^6 CFU/mL (OD_{600} = 0.1).

3.3.3 Screening Antofine against the *S. cerevisiae* haploid deletion mutant library

The sub-lethal concentration (0.20 mg/mL) was used to screen a haploid mutant library of *S. cerevisiae* with deletions in 4847 individual non-essential genes. Briefly, Antofine was dissolved at 0.20 mg/mL in molten 1.5% YPD_{AGAR}. After the medium solidified, a collection of source library plates were used to inoculate the experimental and control plates. The plates were incubated at 30°C and photographed every 24 hours for three days. The photographs were analysed using the ScreenMill Analysis package (Section 2.6) to compare the relative colony size of each mutant between the experimental and control plates.

Of the 4847 mutants tested, 232 were considered to be significantly different (p-value < 0.05) with respect to the growth properties of the untreated control. The 232 mutants were subjected to the same screening test three additional times. Each verification screen was performed at successively higher concentrations. For the first verification screen, the Antofine concentration ranged from 0.20 µg/mL to 0.40µg/mL in 0.05 µg/mL increments; the second screen from 0.40 µg/mL to 0.60 µg/mL in 0.05 µg/mL increments, and finally the third verification from 1.5 µg/mL to 4.5 µg/mL in 1 µg/mL increments. The verification screen resulted in the identification of 30 mutants that consistently displayed a similar phenotype in all three trials. The yeast Open Reading Frame (ORF), common gene name (Gene) and associated function of the gene deleted in these mutants is shown in Table 9.

Table 9: Haploid *S. cerevisiae* mutants sensitive to Antofine

ORF [a]	Gene [b]	ORF/Gene Function [c]
YAL021C	CCR4	Component of the CCR4-NOT transcriptional complex
YBL058W	SHP1	UBX (ubiquitin regulatory X) domain-containing protein
YBL093C	ROX3	Subunit of the RNA polymerase II mediator complex.
YBR081C	SPT7	General transcriptional co-repressor, recruits the SWI/SNF and SAGA complexes to promoters
YBR112C	CYC8	General transcriptional co-repressor, recruits the SWI/SNF and SAGA complexes to promoters.
YCR009C	RVS161	Amphiphysin-like lipid raft protein
YDR176W	NGG1	Transcriptional regulator involved in glucose repression; part of the SAGA complex.
YDR364C	CDC40	Pre-mRNA splicing factor
YDR432W	NPL3	RNA-binding protein
YEL036C	ANP1	Subunit of the alpha-1,6 mannosyltransferase complex
YER068W	MOT2	Subunit of the CCR4-NOT complex
YGL070C	RPB9	RNA polymerase II subunit B12.6.
YGR104C	SRB5	Subunit of the RNA polymerase II mediator complex
YGR167W	CLC1	Clathrin light chain.
YGR240C	PFK1	Alpha subunit of heterooctameric phosphofructokinase.
YGR262C	BUD32	Protein kinase
YHL025W	SNF6	Subunit of the SWI/SNF chromatin remodeling complex.
YIL040W	APQ12	Protein required for nuclear envelope morphology.
YIL053W	RHR2	Constitutively expressed isoform of DL-glycerol-3-phosphatase.
YJL140W	RPB4	RNA polymerase II subunit B32
YJL175W	~	Function Unknown
YJL184W	GON7	Component of the EKC/KEOPS protein complex.
YMR125W	STO1	Large subunit of the nuclear mRNA cap-binding protein complex
YNL199C	GCR2	Transcriptional activator of genes involved in glycolysis.
YNL201C	PSY2	Subunit of protein phosphatase PP4 complex;
YNL220W	ADE12	Adenylosuccinate synthase.
YOL001W	PHO80	Cyclin, interacts with cyclin-dependent kinase Pho85p;
YOL148C	SPT20	Subunit of the SAGA transcriptional regulatory complex,
YPL090C	RPS6A	Protein component of the small (40S) ribosomal subunit.
YPL254W	HFI1	Adaptor protein required for structural integrity of the SAGA complex.

[a] ORF (Open Reading Frame) code that has been deleted in the mutant strain.
[b] Gene name associated with particular ORF
[c] Gene product function associated with particular gene.
All Annotations are from the *S. cerevisiae* genome database. http://www.yeastgenome.org/

3.3.3.1 Antofine perturbs processes involved in transcription and RNA processing.

The thirty ORFs identified in the screen were imported into the *Saccharomyces* Genome Database Analysis tool "Gene Ontology Slim Mapper" which groups gene annotations into broad categories (http://www.yeastgenome.org). This analysis suggested that 23 of the 30 genes are associated with a function in the nucleus; 15 of the 30 are associated with functions in the cytoplasm; and 5 of the 30 are associated with membrane or cytoskeleton function.

To further understand the relationship between these genes and Antofine, the thirty genes were subjected to mapping by GeneMania (http://www.genemania.org/), an online resource that uses a large set of functional association data to display and map relationships between genes and proteins. According to GeneMania, the majority of the selected genes are associated with transcription regulation. For example, six of the genes (CDC40, STO1, RPB4, NPL3, CCR4, MOT2, SPT20) are associated with mRNA metabolic processing; four (SPT20, NGG1, SPT7, HFI1) are associated with the SAGA complex (a transcriptional co-activator of environmental stress response genes), and five (SRB5, SNF6, CYC8, ROX3, GCR2) are transcription regulators (Figure 12).

This observation was supported when this same collection of genes was input into the Saccharomyces Genome Database Gene Ontology Term Finder analysis tool (http://www.yeastgenome.org). According to Gene Ontology Term Finder, 40 % (12 of 30) of the query genes (CCR4, ROX3, CYC8, NPL3, MOT2, SRB5, BUD32, SNF6, RPB4, GON7, GCR2, PHO80) have been associated with regulation of gene expression; this is in contrast to the 8.6% (619 of 7168) ($p = 7.8 \times 10^{-4}$) of the currently

annotated *S. cerevisiae* genome to which gene expression regulation has been associated. In addition, 13% (4 of 30) of the query genes belong to the SAGA complex (SPT20, NGG1, SPT7, HFI1), which itself comprises only 0.3% (20 of 7168 genes) ($p = 6.11 \times 10^{-5}$) of the currently annotated *S. cerevisiae* genome.

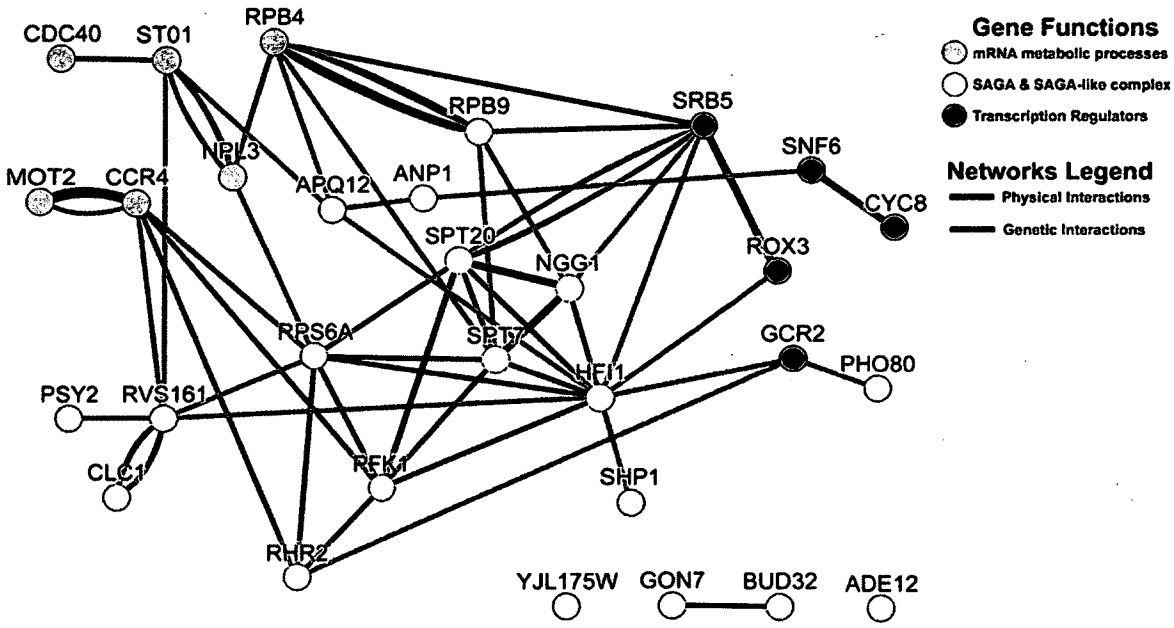

Figure 12: Relationship between the *S. cerevisiae* mutants sensitive to Antofine

Chapter 4 Discussion

Fusarium head blight is a problem faced by cereal growers worldwide; the disease is responsible for millions of dollars in losses annually in North America alone. In addition, there are few products available to growers to combat this problem. The purpose of my project was to discover novel, environmentally friendly fungicides that could be used against to control Fusarium Head Blight disease. The project was composed of two distinct parts.

The first part of the project was the development of a high-throughput screening method by which large numbers of compounds could be tested for efficacy against *F. graminearum*. More than 500 compounds from a commercially available chemical library, and 23 plant extracts were tested. The screen uncovered 26 compounds and 15 plant extracts with strong inhibitory qualities against *F. graminearum* growth in liquid culture.

The second part of the project was an attempt to gain some measure of understanding as to the mode of action of the compounds identified during the chemical screen. To achieve this purpose, one of the readily available and potent chemical compounds, "Antofine" was screened against a yeast haploid deletion library. Through this screen I hypothesize that Antofine is most active against putative pathways involved in transcription control, RNA processing, and also has some influence over the SAGA pathway.

4.1 Structure/function of chemical compounds

Thirty one of the thirty seven compounds identified from the chemical screen were classified into seven distinct groups, six of which were based upon a common backbone, and the seventh consisted of compounds that were unique.

The first group was comprised of compounds which are based upon a benzimidazole backbone attached to a menthol group as one side chain (R1), while the second side chain (R2) varies (Figure 3). Benzimidazole compounds are already known to have biological effects, and several are already registered as commercially available pesticides (FRAC, 2011). In animals, benzimidazole compounds have been demonstrated to block glucose uptake and to interfere with energy metabolism (Danaher *et al*, 2007). The primary mode of action ascribed to benzamidizole is that it interferes with the proper formation of microtubules. Since many cellular processes are depended upon proper microtubule formation, inhibition eventually results in cell death (Danaher *et al*, 2007). Recent reports from China suggest emergence *F. graminearum* strains resistant to currently registered benzimidazoles (Chen and Zhou, 2009; Chen *et al*, 2009). It would be interesting to see if any of the benzamidizole-derived compounds identified in my screen will retain activity against those *Fusarium* strains currently developing benzimidazole resistance.

The second group is composed of compounds which are derivatives of or contain a gossypol backbone. Gossypol (Figure 4) is a polyphenolic that can act as a Schiff base, and is believed to act by cross linking proteins through a reaction between the aldehydic gossypol groups and the amino groups of proteins (Wang *et al*, 2009). Due to its reactive nature, gossypol has been attributed a variety of biological effects, including antioxidant,

contraceptive, anticancer, antiviral, antiparasitic, antiprotozoan, and antimicrobial properties (Wang *et al*, 2009). The reason for the differences between the antifungal properties observed in the group 2 compounds is not precisely known. It is possible for example that the side chains actually take part in the inhibitory effect, or they may be inert and when removed, allow the gossypol backbone to react with other cell components. Alternatively, the side chains many not be removed at all, but instead exert some form of influence upon the reactivity of other reactive groups of the molecule.

The inhibitory effects of the fourth group (Figure 6) are interesting. Since only one of the three (ST009866) demonstrated strong inhibition of growth at 0.0125 mg/mL (ten-fold less than the highest concentration tested), it is conceivable that the side chains play a very important role. The only major difference between ST009866 and the other two is that the attached furan ring is a five member ring, while the other two contain six member rings. In addition, although ST014054 did not meet the necessary criteria to be considered a suppressor, it did demonstrate a greater amount of growth reduction than ST009867. Both ST009866 and ST014054 contain electron rich rings in their side chains, while ST009867 contains a relatively electron poor ring as its side chain (McMurray, 2004). These particular compounds may perform their mode action by being electron donors rather than electron acceptors. Therefore, the increased reactivity of ST009866 is likely due to a combination of a smaller electron rich ring providing greater reactive potential than the larger electron poor ring of ST009867.

The seventh group (Figure 9) included a compound belonging to the chalcone family (ST001473) and an alkaloid (Antofine) with strong inhibitory properties against *F. graminearum*. The compound 4-chlorochalcone, and Antofine, have been shown to be inhibitory against micro-organisms, and shall be discussed in greater detail below.

Chalcones and their derivatives are open chain flavonoids that play a wide variety of biological roles in plants, and have already been established to act as defensive molecules (Batovska and Todorova, 2010). Chalcones have properties such as; antioxidant, cytotoxic, anti-cancer, antimicrobial, antiprotozoal, that make them useful in a variety of medicinal roles as well as having antihistaminic and anti-inflammatory qualities (Batovska and Todorova, 2010). Of particular interest, is that chalcones also have been proved to be potent antifungal compounds (Boeck *et al*, 2005; Lahtchev *et al*, 2008; López *et al*, 2001). The principal mode of action of chalcones as antifungal agents has been suggested to be direct interaction with proteins (Lahtchev *et al*, 2008). The ketone group of chalcone acts as the electron acceptor in a Michael reaction and binds to the thiol groups of the amino acid cysteine in proteins (Lahtchev *et al*, 2008; Batovska and Todorova, 2010). In fungi, the principle proteins affected by chalcones are those associated with the cell wall (Boeck *et al*, 2005; Lahtchev *et al*, 2008; López *et al*, 2001). The chemical potency of chalcones is associated with the identity of the substituents bound to the rings of the molecule. In general electron withdrawing groups such as halogens (F, Cl, Br), or nitro groups (NO_2) increase potency, while electron donating groups such as alcohols, or amines decrease potency (Lahtchev *et al*, 2008).

4.1.1 Side chains contribute to potency

Our evidence suggests that side chains of compounds play an important role in the compound's potency. Lipinski *et al* (2001) suggested five basic criteria that any compound under consideration for use as an oral drug in humans should follow. Lipinski's "rule of five" states that a drug will be poorly absorbed or have a low permeability if the compound has more than 5 H-bond donors, 10 H-bond acceptors, has a molecular weight greater than 500 daltons, and the calculated Log P (partition constant) is greater than 5 (Lipinski *et al*, 2001). Although these qualities may not play as large a role when seeking fungicidal compounds, they do provide a possible framework for selecting compounds for efficacy testing. For example, a non-polar compound when applied may readily diffuse through the phosopholipid membrane, and will resist being washed away during the first rainfall; however for convenience and better control of application by spray, many commercial fungicides are prepared and dissolved in water, so a strongly non-polar compound may be difficult to apply without a surfactant or other adjutant.

Side chains will also modify the potential of the active group in the molecule. The reactive sites of molecules fall into two basic categories, electron acceptors (electrophile) and electron donors (nucleophile). Side chains also have two basic categories, electron donating groups (ED) which provide electrons towards the reactive site, and electron withdrawing groups (EW) which draw electrons away from the reactive site. An ED coupled with an electrophile, or an EW group coupled with a nucleophile will often lower the reactivity of the reactive site, while the opposite; an ED coupled with a nucleophile, or an EW coupled with an electrophile will increase the reactivity of a the reactive group.

For example, chalcone, the molecule upon which 4-chlorochalcone is based will change reactive potential depending upon which side chains are associated with the two aromatic rings (Figure 9) (Boeck *et al*, 2005; Lahtchev *et al*, 2008; López *et al*, 2001).

Different side chains may also be responsible for the physical interactions of a compound with its target. For example, a particularly long side chain may be responsible for steric hindrance and prevent the compound from fitting into and reacting with its target site. Alternatively a longer side chain may be necessary for the association between the compound and its target to properly orient and react. For example, in group 5 (Figure 7) ST012844 demonstrated a ten-fold greater efficacy than ST012842, and yet the only difference is the identity of the side chain. It is quite possible that the side chain itself is the reaction site, however ST012842 has a large bulky acridine group that can only rotate about the nitrogen to which it is bonded, while ST012844 has a phenazone group that is not only smaller, but has two bonds around which the parts of the side chain can rotate in accommodation.

4.1.2 Advantages of using a chemical library

The initial part of the project involved screening both a commercially available chemical library, and a series of natural extracts. Screening a library of pure compounds has certain advantages over working with a set of crude plant extracts, one of which is that the concentration and identity of each compound in the library is known. This allows many compounds to be screened simultaneously at a defined concentration, and the effects of compounds with similar structures can be easily compared to one another. In contrast, a crude natural extract is a combination of many compounds, and therefore, the

exact quantity of each is often unknown and the extract composition will often vary between each extraction.

One of the main advantages of a chemical library is that it provides the researcher with insight as to which modifications to the back bone structure will provide the greatest effect. For example, in group 1 the only difference between ST003705 and ST003707 is the addition of an ether linkage in the R2 side chain, however there is a ten-fold difference in efficacy (ST003707 is more efficacious) (Figure 3). In addition, in the same group the only difference between ST003704 and ST003705 is the exchange of bromine for the methyl group on the R2 side chain, but ST003704 is twice as potent (Figure 3). So by testing a wide variety of compounds and noting the differences in potency provides guidance as to which modifications to attempt and which to avoid, thereby "optimising" the compound for its intended effect.

4.2 Antofine and its mode of action

Antofine is known for its antifungal compound and phytotoxic properties (Gibson *et al*, 2001). The compound belongs to the phenanthroindolizidine class of alkaloids, which have been associated with suppression of DNA and protein synthesis, the inhibition of dihydrofolate reductase, the induction of apoptosis in cancer cell lines, and the inhibition of nuclear factor-kappaB (NF-κB) activation (Min *et al*, 2010). However, to date the exact mode of action has not yet been discerned, and part of this project was an effort to provide some idea of the method by which Antofine influences cellular growth. Antofine was screened against a haploid single knockout library of ~ 5000 *S. cerevisiae* strains; each mutant strain has a different non-essential gene deleted. This screen enabled us to identify pathways that are perturbed by Antofine.

A classical method of determining relationships between genes is to look for combinations of mutations or gene deletions which in combination are lethal, but individually have little effect on the organism's growth, a so called synthetic lethal assay (Stockwell, 2004). By determining which combinations of mutation do not support growth, it becomes possible to determine genetic interactions and map affected networks and pathways.

Analogous to the synthetic lethal assay, Antofine was used to perform a synthetic dosage screen assay. In this scenario a library of mutants was grown in the presence of Antofine. When Antofine disrupts the function of its target protein, and if the deleted gene in the library participates in the final outcome (death of the yeast strain), then we can attribute the phenotype to the interaction of the target of Antofine to the disrupted gene (Stockwell, 2004). By analysing the mutants that are affected by Antofine it was possible to identify which pathways or processes are being perturbed by Antofine. The outcome of this screen resulted in the identification of 30 genes belonging to three groups that are likely involved in Antofine action. The genes related to the SAGA complex, the genes related to the CCR4-Not complex, and the third group, belonging to the RNA polymerase II subunits.

The SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex is a highly conserved transcriptional co-activator responsible for the activation of approximately 10% of the genes yeast use to respond to environmental stresses (Daniel & Grant, 2007; Samara and Wolberger, 2011). The ~1.8 MDa complex is made up of 21 proteins (Samara and Wolberger, 2011) and is functionally redundant to the general transcription factor TFIID, is generally targeted to promoters by DNA-bound transcriptional activators,

and can interact directly with multiple activators through one of its subunits, (Tra1) to stimulate in vitro transcription in a manner dependent upon acetyl-CoA (Daniel & Grant, 2007). Different combinations of subunits are required to activate different distinct functions, and some subunits have been attributed different functions when different locations are targeted. Some subunits, such as Spt7 and Ubp8 are critical to the function of the complex since their elimination will reduce the protein levels of other components (Daniel and Grant, 2007; Samara and Wolberger, 2011). The SAGA complex has also been associated with acetylation and de-ubiquitination of histones, thereby associating the SAGA complex with roles in transcription control, gene silencing, and mitotic and meiotic cell growth processes (Daniel & Grant, 2007).

The CCR4-Not complex is a unique, essential, multi-subunit complex that is highly conserved across the eukaryotic kingdom. In yeasts, it consists of nine core subunits and regulates gene expression to control cellular function on many different levels (Collart and Panasenko, 2012). The CCR4-not complex has both the enzymatic activities of ubiquitination, provided by the Not4 subunit, and deadenylation provided by the Ccr4 subunit (Collart and Panasenko, 2012). A myriad of functionalities have been suggested to be associated with the CCR4-not complex, including post-translational modification of proteins, regulation of protein arginine methylation, interaction with the TFIID transcription factor, and interaction with the SAGA acetyltransferase complex (Collart and Panasenko, 2012). It has also been suggested that CCR4-not directly promotes transcriptional elongation, and binds directly to RNA polymerase II elongating complexes to stimulate the reactivation of arrested RNA polymerase II (Collart and Panasenko, 2012). The complex has also been implicated in regulating

mRNA synthesis, translation, degradation, and plays a role in response to DNA damage (Collart and Panasenko, 2012).

Finally, RNA polymerase II (RNAP II) has the primary function of synthesizing the precursors of mRNAs, most snRNA and microRNAs in eukaryotes (Sims *et al*, 2004). Twenty four out of 30 genes perturbed by Antofine in *S. cerevisiae* have homologues present in *F. graminearum*, including those belonging to RNA polymerase II. Interestingly the regulation of genes linked to small or micro RNAs has not been studied in this fungus.

Of obvious value would be to determine the targets of the chemical compounds. One method that could be used to find chemical targets would be to take advantage of haploid insufficiency; in which the loss of function of one gene copy leads to the observation of an abnormal phenotype. Under normal conditions a single copy of a gene is sufficient to prevent such abnormal phenotypes from being observed. However the influence of an inhibitory drug further reduces the functionality of the target gene product, and does result in the observation of an abnormal phenotype (Giaever *et al*, 1999). Therefore, by screening a *S. cerevisiae* diploid heterozygous knock-out library with a nominally sub-lethal concentration of drug allows direct drug targets to be suggested. In addition, unlike the haploid knock-out library, the essential genes are also represented, allowing chemical effects upon the gene products of the entire genome to be assessed.

4.3 Concluding remarks

My project involved the development of a method by which crude extracts and purified compounds were screened against *F. graminearum* in an effort to uncover new potential fungicides used to control the pathogen.

The methodology employed in my thesis is not limited to study of a single pathogen like *F. graminearum*, but can be adapted to other pathogens that can be cultured in the laboratory. The strain of *F. graminearum* used in this project (ZTE-2A) had been transformed to constitutively express GFP, so that the rate of growth of the organism could be easily monitored by GFP fluorescence. Since transformation with GFP or other related molecules is a routine laboratory procedure, and GFP is not considered to be detrimental to the expressing organism, any transformable fungal strain could be subjected to the same screening methodology.

4.3.1.1 Chemical genomics: part of the tool box

Chemical genetics uses small molecules (chemicals) to perturb or alter the expression and/or function of biological systems in a conditional, rapid, and reversible manner thereby providing a method by which systems can be studied (Spring, 2005). The use of a chemical rather than genetic approach allows dosage modification and thus allows us to study threshold effects of gene expression. The results of chemical inhibition are also often rapid, allowing for dynamics studies. In contrast a genetic knockout can only present the results of a steady state condition (Spring, 2005; MacBeath, 2001).

Some metabolic processes and pathways are not essential for the survival of the organism, but may contribute to its fitness. For example, in *F. graminearum* encodes many genes resulting in the production of secondary metabolites; including the mycotoxin DON, which confers increased virulence to the pathogen. In addition to mycotoxins, *F. graminearum* also encodes a number of plant-cell wall degrading enzymes that are important in the invasion and digestion of plant tissues (Cuomo *et al*, 2007). These include cutinases, enzymes that can hydrolyze cutin polyesters found on the outer surface of plants; pectate lyases, to digest pectin, an essential component of plant cell walls; and xylanases, an enzyme which degrades xylan, a major hemicellulose component of monocot cell walls (Cuomo *et al*, 2007). Chemical inhibition of any of these processes would give added advantage to study this organism at various stages of infection and furthermore, offer novel compounds for curtailing the disease symptoms in the field.

The ultimate goal of chemical genomics is to discover or produce one or more ligands that bind specifically for every protein in a cell, tissue or organism (MacBeath, 2001). One method of achieving this goal is to utilise the same strategy employed in forward genetics, screening large numbers of chemical compounds, selecting those compounds which generate a phenotype of interest, and then determining the chemical mode of action (MacBeath, 2001).

4.4 Future experiments:

Since many of the genes highlighted in the haploid screen are conserved between *S. cerevisiae* and *F. graminearum*, two experiments that could be performed are to functionally complement the haploid *S. cerevisiae* knock-out with the *F. graminearum* homologues. Conversely the homologous genes to the targets identified in *S. cerevisiae* could be deleted in *F. graminearum* in an attempt to either elicit an increase in sensitivity or confer immunity to the drug. In either case the results would help to provide evidence to confirm the identity of the targets and pathways affected.

Although the efficacy of Antofine and the other chemical compounds cited in this study has been tested *in vitro* against *F. graminearum*, the efficacy *in vivo* has not yet been established. So a logical experiment to perform would be to test Antofine and the other inhibitory chemicals to prevent Fusarium head blight disease

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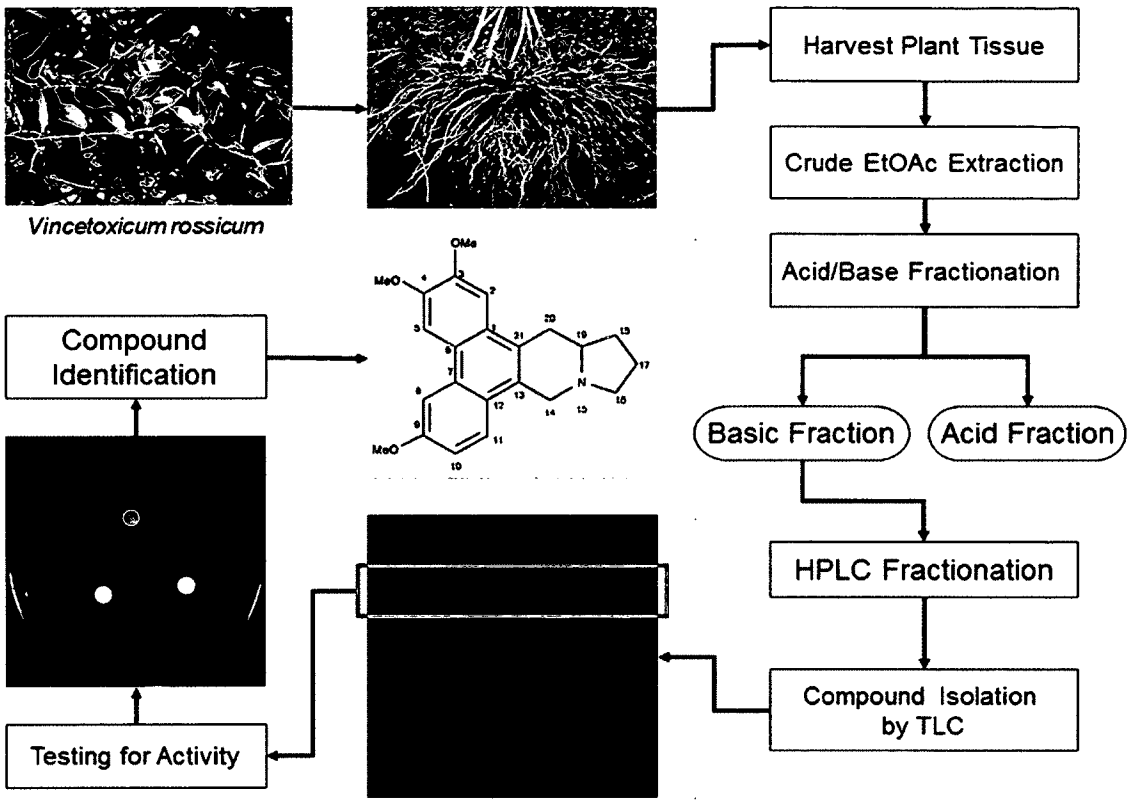
Appendix



Appendix Figure 1: Antofine extraction procedure

Media Recipes

CMC Broth Recipe (Carboxy-Methyl-Cellulose)

NH_4NO_3:	1 g
KH_2PO_4:	1 g
$MgSO_4 \cdot 7H_2O$:	0.5 g
Yeast Extract:	1 g
Carboxymethylcellulose:	15 g
Water:	Bring up to 1L

YPD Broth Recipe (Yeast Extract Peptone Dextrose)

Peptone:	20 g
Yeast Extract:	5 g
Dextrose:	10 g
Water:	Bring up to 1L

GYEP Broth Recipe (Glucose-Yeast Extract-Peptone)

NH_4Cl:	3 g
$MgSO_4 \cdot 7H_2O$:	2 g
$FeSO_4 \cdot 7H_2O$:	0.2 g
KH_2PO_4:	2 g
Peptone:	2 g
Yeast Extract:	2 g
Malt Extract:	2 g
Glucose:	20 g
Water:	Bring up to 1 L