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Detection of Siderophores in Selected Species of Rhizoctonia Solani Species Complex

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DETECTION OF SIDEROPHORES IN SELECTED SPECIES OF *RHIZOCTONIA SOLANI*

SPECIES COMPLEX

A Thesis

Submitted to the School of Graduate Studies and Research

in Partial Fulfillment of the

Requirements for the Degree

Master of Science

Alexander Wiredu

Indiana University of Pennsylvania

May 2015

Indiana University of Pennsylvania
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Title: Detection of Siderophores in Selected Species of *Rhizoctonia solani* Species Complex

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The pathogenicity of microorganisms has been attributed to specific biomolecules produced by these microorganisms, Siderophores are low molecular weight, high affinity ferric iron chelators, which are synthesized and secreted by many microorganisms in response to iron deprivation. These compounds solubilize and bound iron and transport it back into the microbial cells. My goal was to find out if Siderophores are responsible for the pathogenicity of *Rhizoctonia solani,* which is a soil borne plant pathogen. To study this, five isolates of *Rhizoctonia solani* were selected depending on their differences in virulence, each isolate was cultured and the resulting metabolic footprints were examined using chromatographic, spectrophotometric, and chemical methods. The amount of Siderophore produced by each selected isolate was detected and quantified. It was possible to draw correlation that, the amount of Siderophore produced is directly proportional to the pathogenicity of the isolates.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

%	Percentage
ºC	Degree Centigrade
ºF	Degree Fahrenheit
GC	Gas chromatography
MS	Mass spectroscopy
MALDI	Matrix-assisted laser desorption/ionization
TOF	Time of flight
ESI	Electrospray ionization
D	Dextro
L	Levo
R	Rectus
S	Sinister
α	Alpha
β	Beta
δ	Delta
PAA	Phenylacetic acid
Ka	Binding constant
M^{-1}	Per mole
NPS	Nonribosomal peptide synthetase
DNA	Deoxyribonucleic acid
pH	Potentia hydrogenii
Fe	Iron

ABBREVIATIONS

FDA	Food and drug administration
+	Plus
Vol	Volume
CAS	Chrome Azurol S
mM	MilliMolar
ml	Milliliter
$Fecl_3$	Iron (iii) Chloride
mg	Milligram
H_2O	Water
HCl	Hydrochloric acid
MES	2-(N-morpholino) ethane sulfuric acid
HDTMA	Hexadecyltrimethylammonium
KOH	Potassium Hydroxide
Mins	Minutes
L	Liter
M	Molar
HPLC	High performance liquid chromatography
PDA	Potato dextrose agar
UV	Ultra-violet
nm	nanometer
&	and

CHAPTER 1

INTRODUCTION

Rhizoctonia solani

Rhizoctonia solani (teleomorph: *Thanatephorus cucumeris*.) is a plant pathogenic fungus, with a wide host range and worldwide distribution, it causes diseases on a lot of plants including cotton, tobacco, tomato and potato. The Fungus is found in the upper layers of the soil and can spread rapidly at temperatures below 68ºF. It does not produce spores and it exist as thread-like growth on plants or in culture, it attacks its hosts in their juvenile stages of development which are typically found in the soil, hence it is considered as a soil-borne pathogen (A. Ogoshi, 1987). The fungus *Rhizoctonia solani* complex is a taxonomic entity composed of morphologically similar groups, which share similar characteristics such as multinucleate cells, production of sclerotia, and lack of Conidia (asexual spores).

The identification of *Rhizoctonia solani* has been based on the ability of their hyphae to anastomose (hyphal fusion between compatible strains). *Rhizoctonia solani* is the most widely recognized species of the genus Rhizoctonia, it is considered a basidiomycete, it does not produce asexual spores (conidia) although it is considered to have an asexual life cycle. It occasionally produces basidiospores (sexual spores) on infected plants, *Rhizoctonia solani* reproduces asexually and exist as vegetative mycelium and /or sclerotia. The mycelium consists of hyphae, partitioned into individual cells by a septum containing a dough-nut shaped pore, this pore allows for the movement of protoplasmic materials from cell to cell (B. Sneh, 1996).

The pathogen prefers warm wet weather, and outbreaks usually occur in the early months of summer, it can survive in the soil for many years in the form of sclerotia, these sclerotia can travel by means of wind, water or soil movement between host plants (Cubeta and Vilgalys, 1997). The fungus is attracted to the host plants by chemical stimuli released by a growing host plant and/or decaying plant matter. The most common symptom of Rhizoctonia solani disease is called "dumping of ", which is characterized by non germination of affected seeds, other symptoms include seed decay, stem cankers, fruit decay and foliage diseases (Cubeta and Vilgalys, 1997).

Metabolite Profiling in Fungal Pathology and Taxonomy

Mycologists have traditionally relied on morphological features as their basis of classification, however absence of sexual or sporulating stages and similarities in morphology of a number of fungi species, possess problems in identification and classification. This has led to the use of chemical methods to complement already existing taxonomic methods. The classification of *Rhizoctonia solani* species complex has primarily been done by hyphal anastomosis testing, and molecular methods (Cubeta and Vilgalys, 1997), molecular methods using ribosomal DNA sequence analysis of *internal transcribed spacer* (Sharon et al., 2006), and DNA Bar-coding. Chemotaxonomy based on metabolite profiling (Allen et al., 2003; Larsen et al., 2005; Frisvad et al., 2008) have currently been employed in fungal taxonomy, ecology and pathology. The concept of hyphal anastomosis implies that, isolates of Rhizoctonia that have the ability to recognize and fuse (anastomose) with each other are genetically related, whereas those that lack this ability are considered to be genetically unrelated. This criterion has been used to place isolates of Rhizoctonia into taxonomically distinct groups called anastomosis groups.

Currently there are 14 known anastomosis groups (AGs) of *Rhizoctonia solani* (AG1 to AG13 and AGB1). Seven of the 14 AGs (AGs1-4, AG6, AG8 and AG9) are further subdivided into subgroups based on frequency of anastomosis, pathogenicity, biochemical characteristics, cultural appearance and thiamine requirements (Cubeta and Vilgalys, 1997).

The most common way used in hyphal anastomosis involves pairing two isolates on a glass slide and allowing them to grow together, the area of merged hyphae is stained and examined microscopically for the resulting hyphal interaction(s). Currently a lot of research is being done in the use of common metabolites from groups of organisms to study their pathology, ecology, and taxonomy, with taxonomy, the higher the number of common metabolites that a set of organisms share, the more closer they are related to each other, this new field of study is called chemotaxonomy. And it is a high throughput methodology that complements existing molecular approaches of classification. The use of metabolomics in Fungi classification is a relatively new field and quite a few chemotaxonomic studies have been used to investigate the identification and classification of Fungi including *R. solani* species complex (Aliferis et al., 2011). Gas Chromatography and Mass Spectrometry (GC/MS) have been used in the analysis of metabolites in the chemotaxonomy of various genera and species of Fungi. GC/MS analyses were used in chemotaxonomic discrimination among the genera T*olypocladium*, *Beauveria* and *Paecilomyces* (Kadlec et al., 1994).

Chemotaxonomic discrimination of species of *Aspergillus* was made possible by the application of Matrix-Assisted Laser Desorption /Ionization (MALDI)-time of flight-MS (MALD-TOF/MS) (Hettick et al., 2008). Direct electro spray/MS (ESI/MS) applied singly or in combination with GC/MS was used to classify species of *Penicillium* and was capable of discriminating between mutants of *Saccharomyces cerevisae* (Smedsgaard and Nielsen, 2005).

Filamentous fungi produce a complex and broad range of metabolites (Aliferis and Jabaji, 2010) which is made up of endo-metabolome (intracellular primary and secondary metabolites) and the exo-metabolome (the metabolites excreted into the growth media). The unspent growth media together with the exo-metabolome forms the metabolic footprint which is profiled for the purpose of fungal pathology, ecology, and taxonomy (Frisvad et al., 2008), this branch of science is called metabolomics. The metabolites that are isolated and identified from the metabolome serves as signatory metabolites and these include siderophores, amino acids, carboxylic acids, fatty acids and carbohydrates. Examples of amino acids that have been isolated and identified are glycine, L- serine, L- valine, L- isoleucine, and L- threonine, fatty acids that have been isolated and identified include palmitic acid, oleic acid, linoleic acid and stearic acid. Glycerol, erythrose, D-glucitol, D-ribo-hexitol, and α-α-trehalose are the common carbohydrates that have been isolated and identified. Phenylacetic acid (PAA), fumaric acid, malic acid, isocitric acid and L-aspartic acid are among the carboxylic acids that have been isolated and identified. Examples of fungal siderophores that have been isolated and identified are Ferrichromes, Coprogens and Triacetylfusarinine C.

The pathogenicity of microorganisms has been attributed to the presence of specific biomolecules produced by these organisms (Calderone and Fonzi, 2001), phenylacetic acid (PAA) is believed to be contributing factor to the pathogenicity of *Rhizoctonia solani*, correlation analyses revealed that isolates of *Rhizoctonia solani* that produce high PAA and its derivatives caused high mortality on tomato seedlings (Bartz et al., 2012). However to the best of my knowledge, this current study is among the pioneering works to study siderophores and their effect on the pathogenicity of *Rhizoctonia solani*.
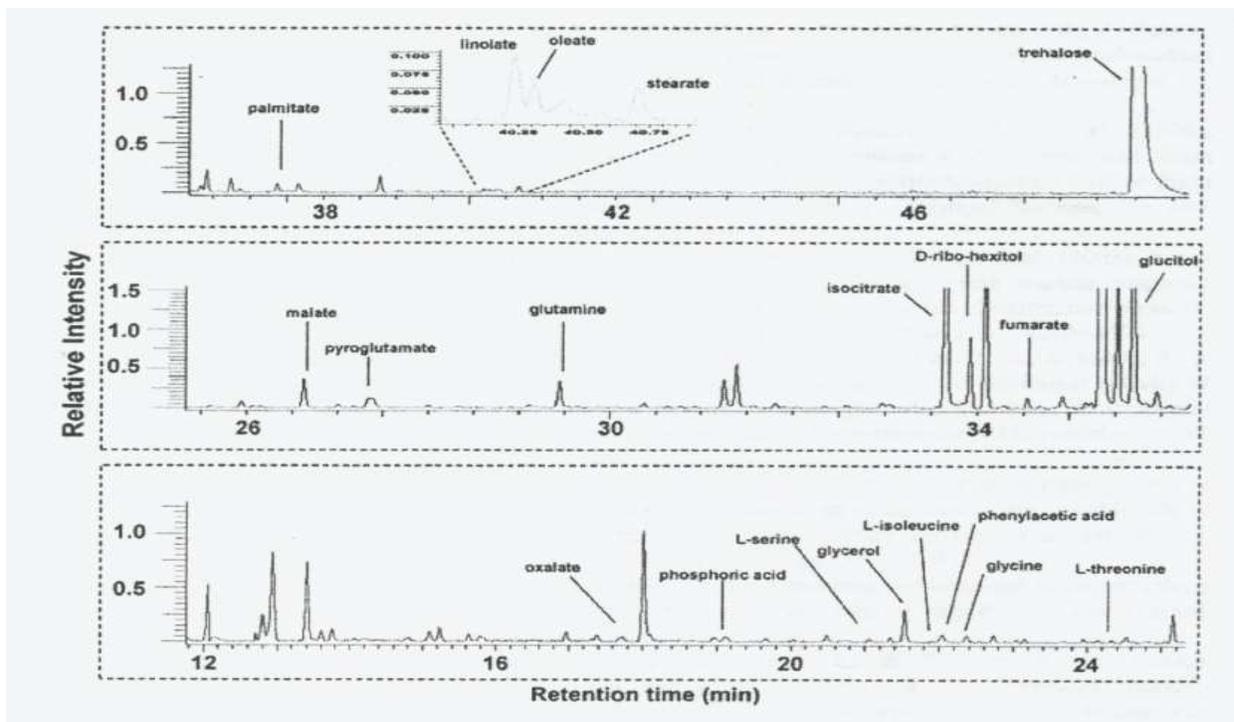
Figure 1. Ion chromatogram of selected signatory identified fungal derived metabolites. The figure shows metabolites that have been identified as biomarkers in the chemotaxonomy of R. solani. Source: (K.A. Aliferis et al., 2011).

The most common carboxylic acids being used in fungal metabolomics include, Phenylacetic acid (PAA), Malic acid, Fumaric acid, isocitric acid and L-aspartic acid.

a   b
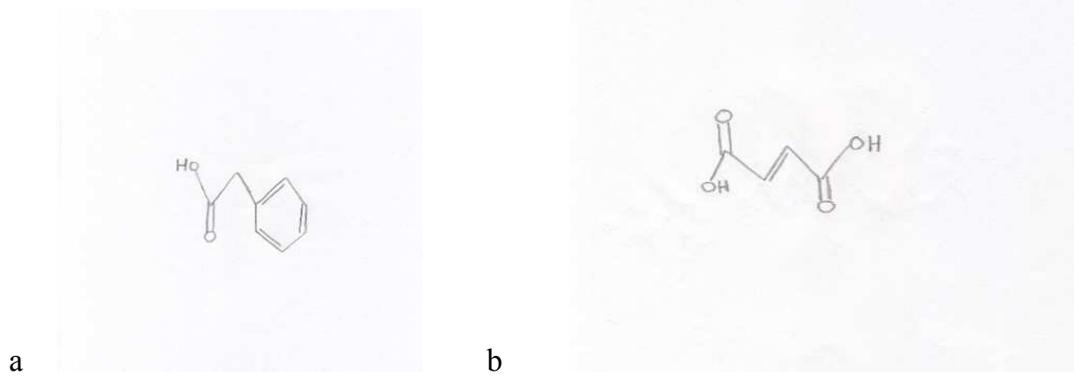
Figure 2. (a) Phenylacetic acid (b) Fumaric acid. This figure depicts the chemical structures of phenylacetic acid and fumaric acid.

The common Amino acids being used in fungal metabolomics include Glycine, L-serine, L-valine, L-isoleucine and L-threonine.

a   b
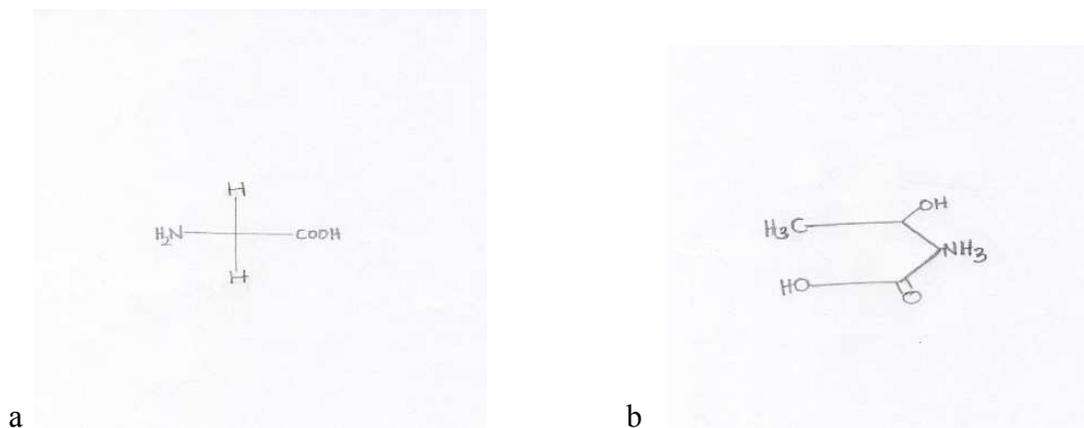
Figure 3. (a) Glycine (b) L-Threonine. This figure illustrates the chemical structures of glycine and L-Threonine.

The common carbohydrates used in fungal metabolomics include, α-α-trehalose, Erythrose, D-ribo-hexitol, Glycerol, and D-glucitol.

a   b
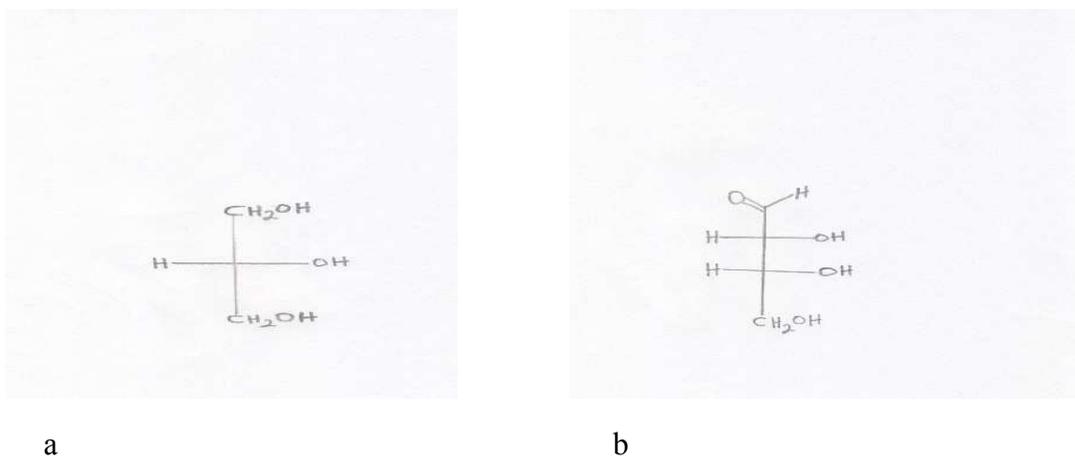
Figure 4. (a) α-α-Trehalose (b) D-Erythrose. This figure shows the chemical structures of α-α-trehalose and D-Erythrose.

The common Fatty acids being used in fungal metabolomics include, Oleic acid, Palmitic acid, Stearic acid and linoleic acid
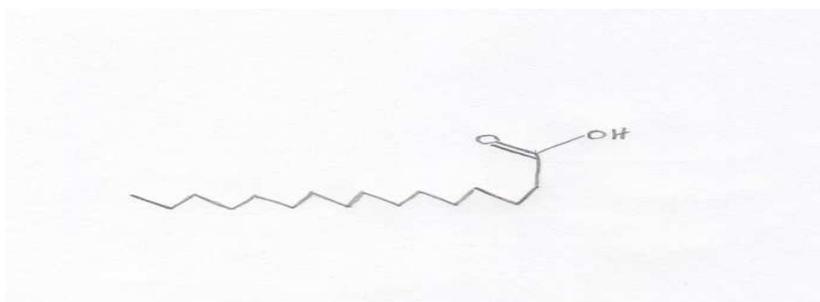
Figure 5. Palmitic acid. The figure shows the chemical structure of palmitic acid.

Biochemistry of Siderophores

Siderophores are low molecular weight high affinity ferric iron chelators which are synthesized and secreted by many microorganisms in response to iron deprivation. These compounds solubilize and bound iron and transport it back into the microbial cell, usually through specific membrane receptors. The most common Fungal Siderophores includes Ferrichromes, Coprogens and Triacetylfusarinine C. Some Siderophores have the ability to remove iron from mammalian-iron binding proteins (J. B. Neilands, 1995).

The ability of siderophores to express these iron transport has been associated with microbial pathogenicity (J. H. Crosa, 1989). Siderophores also can act as intracellular iron storage compounds, they can as well suppress the growth of other microorganisms. Siderophores can complex other metals aside iron, in particular the actinides. Because of their metal binding ability, they have potential application in medicine, reprocessing of nuclear fuel, remediation of metal-contaminated sites and industrial waste management. Most siderophores are classified as either hydroxamates or phenolates-catecholates with the exception of a very few (Dave and Dube, 2000).

Siderophores are transported to the extracellular environment by efficient export machinery, on the other hand after iron sequestration in the environment, the iron-siderophore complex is captured by specific membrane receptors to be transported into the cell, however these siderophore transport mechanisms vary among different microorganisms (Zheng and Nolan, 2012). Currently a lot of studies have been done on siderophores and this has led to its applications in the pharmaceutical and medical fields. Desferrioxamine B (marketed under the trade name Desferal) is an FDA-approved iron chelator for the treatment of iron-overload diseases such as hemochromatosis and thalassemia (J. B. Neilands, 1995).

Siderophores, and siderophore mimics are being used as cancer therapeutics due to the basic fact that cancer cells require high iron concentrations for proliferation (Zheng and Nolan, 2012). Sideromycins are siderophore-drug conjugates that are being applied as "trojan horse" antibiotics, which are recognized and transported into recipient bacterial cells, by siderophore uptake machinery, an example is albomycin (Zheng and Nolan, 2012).

Figure 6. Triacetylfusarinine C. The figure shows the chemical structure of triacetylfusarinine C.

CHAPTER 2

LITERATURE REVIEW

Biomarkers

A biomarker is basically a measurable indicator whose presence is indicative of a phenomenon, in medicine biomarkers are use to indicate the presence, severity and monitoring of diseases, in pharmacology, biomarkers are used to study drug target identification and drug response. Biomarkers are also applied in other fields such as biofuels, forensics and security. Biomarkers that are being employed in fungal pathology, ecology, and classification include siderophores, fatty acids, amino acids, carbohydrates and carboxylic acids, the most common ones are ferrichromes, coprogens, triacetylfusarinine C, palmitic acid, oleic acid, stearic acid,, glycine, L-valine, L-serine, L-isoleucine, erythrose, D-ribo-hexitol, glycerol, α-α-trehalose, phenylacetic acid, fumaric acid, isocitric acid and L-aspartic acid (Aliferis et al., 2011).

Siderophores

Siderophores are low molecular weight, high affinity ferric iron chelators, which are synthesized and secreted by many microorganisms in response to low iron levels. These compounds solubilize and bound iron and transport it back into the microbial cell, usually through specific membrane receptors. As naturally occurring chelating agents for iron they are used for the deferrization of patients suffering from transfusion-induced siderosis. A Siderophore from *streptomyces pilosus*, desferrioxamine B is marketed as the mesylate salt, under the trade name Desferal and is advocated for removal of excess iron resulting from the supportive therapy for thalassemia (J. B. Neilands, 1995).

Iron is essential for metabolic processes such as respiration and DNA synthesis, meanwhile the predominant state of iron in aqueous non-acidic oxygenated environments is the Fe^{3+} form, which is not readily available to organism. Siderophores forms soluble Fe^{3+} complexes that can be taken up by active transport mechanisms (J.B. Neilands, 1995). Siderophores can be divided into three main classes depending on the chemical nature of the moieties donating the oxygen ligand for Fe^{3+} coordination, which are either catecholates also known as phenolates, hydroxamates, and carboxylates. Research has shown the existence of mixed ligand type that integrates the chemical features of at least 2 classes into one molecule. An example of the hydroxamates is Ferrichrome which is produced by the fungus *Ustilago sphaerogena*, enterobactin is one example of the catecholates and it is produced by *Escherichia coli*, rhizoterrin is an example of the carboxylates and it is produced by *Rhizopus microsporus*, an example of the mixed ligand siderophore is azotobactin, which is produced by *Azotobacter vinelandii* (Dave and Dube, 2000).

The presence of oxygen in the atmosphere of the earth has resulted in surface iron, existing in forms of low solubility, microorganisms growing under aerobic conditions require iron for metabolic functions such as ATP synthesis, reduction of ribotide precursors of DNA and formation of heme, it has thus become necessary that such microbes have specific molecules that can compete effectively for the iron in the environment. Most siderophores show very high affinity and selectivity for Fe^{3+} with binding constants (K_a) ranging from 10^{30} M^{-1} to 10^{52} M^{-1} (Albrecht-Gary and Crumbliss, 1998). Mycobactin P was isolated in 1949 (Francis et al,. 1949) and since then over 500 siderophores have been discovered and about half of these have been characterized structurally (Zheng and Nolan, 2006).

Nonribosomal peptide synthetase (NRPS) assembly lines are responsible for the biosynthesis of many siderophores (Peuckett et al., 2009), the biosynthesis and transport of siderophores are regulated by repressor proteins that are sensitive to iron concentration, for example, most Gram-negative bacteria produces an iron-uptake regulation protein called Fur. Fur is a Fe^{3+} binding protein whose holo form coordinates to specific DNA regulatory sequences, this results in repression of the transcription of relevant genes when iron levels are high (Bagg and Neilands, 1987).Therapeutic and analytical studies done on siderophores have led to its applications in the pharmaceutical and medical fields. Desferrioxamine B (marketed under the trade name Desferal) is an FDA-approved iron chelator for the treatment of iron-overload diseases such as hemochromatosis and thalassemia (J. B. Neilands, 1995). Siderophores and siderophore mimics are being used as cancer therapeutics due to the basic fact that cancer cells require high iron concentrations for proliferation (Zheng and Nolan, 2012). Sideromycins are siderophore-drug conjugates that are being applied as "trojan horse" antibiotics, which are recognized and transported into recipient bacterial cells, by siderophore uptake machinery an example is albomycin (Zheng and Nolan, 2012).

Such siderophore-antibiotic conjugates usually exhibit β-lactams, fluoroquinolones, and sulfonamides activities. A Mycobactin-artemisinin conjugate has been reported which exhibits activity against *Mycobacterium tuberculosis* and the malaria pathogen (Miller et al., 2011). The exquisite specificity and high affinity of siderophores to the ferric iron is also been tapped in siderophore based strategies for Fe^{3+} sensing and pathogen detection (Zheng and Nolan, 2012).

Siderophores in Microorganisms

Different kinds of siderophores are produced by microorganisms, including fungi, bacteria, actinomycetes, and algae. Bacteria are common inhabitants of metal contaminated sites where they accumulate and immobilize heavy metals, bacteria produce four types of siderophores, salicylates, hydroxamates, catecholates and carboxylates, some siderophore producing bacteria include, *Escherichia coli*, *salmonella*, *klebsiella pneumonia*, *vibrio cholera*, *vibrio anguiliarum*, Aeromonas*, Aerobacter aerogenes*, *Enterobacter, Yersinia* and *Mycobacterium* species (R. Balagurunathan, 2007).

Next to bacteria in terms of microbial siderophore production are fungi, these include, *Aspergillus nidulans, Aspergillus versicolor, Penicilium chrysogenum, Penicilium citrinum, mucor, Trametes versicolor, Rhizopus, Ustilago sphaerogena, Saccharomyces cerivisiae, Rhodotorula minuta* and *Debaromyces* species. Some Algae that produce siderophores are *Anabaena* species. *Anabaena flos-aquae* and *Anabaena cylindrical* produces copper accumulating siderophores. Actinomycetes are aerobic gram positive filamentous bacteria with high guanine + cytosine content and form asexual spores, they are saprophytic in nature and prefer complex substrate for their growth and are able to tolerate high metal concentrations of certain metals, those that produce siderophore include *Actinomadura madurae, Nocardia asteroids,* and *Streptomyces griseus* (R. Balagurunathan, 2007).

Table 1

List of Siderophores from Selected Microbes. Source: ENVIS CENTRE Newsletter Vol. 5, July 2007.

Organisms	Siderophores
Bacteria	
Escherichia coli	Enterobactin
Pseudomonas aeruginosa	Pyoverdin and pyochelin
Aerobacter aerogenes	Aerobacin
Mycobacterium tuberculosis	Mycobactin
Staphylococcus aureus	Aureochelin
Vibrio cholerae	Vibriobactin
Fungi	
Ustilago sphaerogina	Ferriaxamine B
Aspergillus nidulans	Hydroxamate type
Penicillium	Hydroxamate type
Nocardia sp.	Desferrioxamine B

Fungal Siderophores

Most fungi are known to produce siderophores under aerobic conditions during iron limitation, with hydroxamates being the most notable, these siderophores include fusarinines, Coprogens and Ferrichromes. Zygomycetes are however known to produce the carboxylate siderophore rhizoferrin. Fusarinines are made up of two or three cis- fusarinines subunits joined by a labile α-amino ester bond. Two cyclic trimers of cis-fusarinines, fusarinines C and Triacetylfusarinine C have also been identified (J.C. Renshaw et al., 2002).
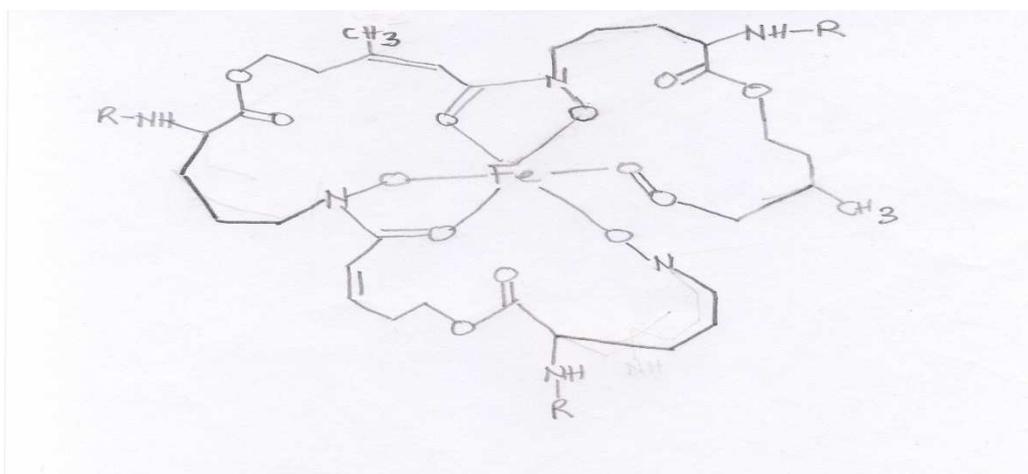
Figure 7. Triacetylfusarinine C. The figure shows the chemical structure of triacetylfusarinine C,

Coprogens consist of trans-fusarinines subunits, they are linear molecules that take either dihydroxamate or trihydroxamate forms, examples are rhodotorulic acid and dimerum acid, the variable R groups or side chains of the Coprogens include hydrogen, methyl, acetyl, anhydromevalonic acid, 4-hydroxyanhydromevalonic acid and Palmitic acid. These side chains alter the hydrophilic nature of the siderophores and hence their solubility in water (J.C. Renshaw et al 2002).
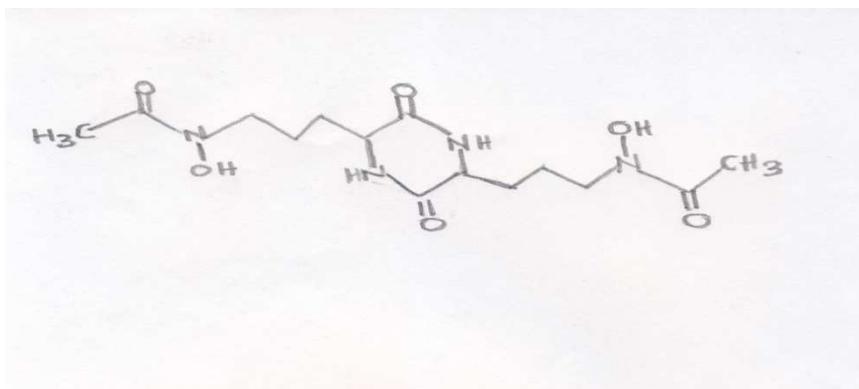
Figure 8. Rhodotorulic acid. The figure shows the chemical structure of rhodotorulic acid.

Ferrichromes are cyclic hexapeptides composed of three, N^δ-acyl-N^δ-hydroxy-L-ornithine, two variable amino acids (alanine, serine or glycine) and a glycine linked by way of peptide bond (Jalal and van der Helm, 1991). Their side chains include acetyl, malonyl, trans-β-methylglutaconyl, transanhydromavelonyl and cis-anhydromevalonyl. Rhizoferrin is a carboxylate siderophore synthesized by members of the Zygomycetes (Thieken and Winkelmann 1992).

Rhizoferrin is composed of diaminopropane symmetrically acylated with citric acid via amine bonds to the terminal carboxylate of the citric acid, both fungi and bacteria produce rhizoferrin, fungi produces only *R, R* rhizoferrin (Drechsel et al., 1992) while a few bacteria produce enantio-rhizoferrin, the *S, S* rhizoferrin (Munzinger et al., 1999).
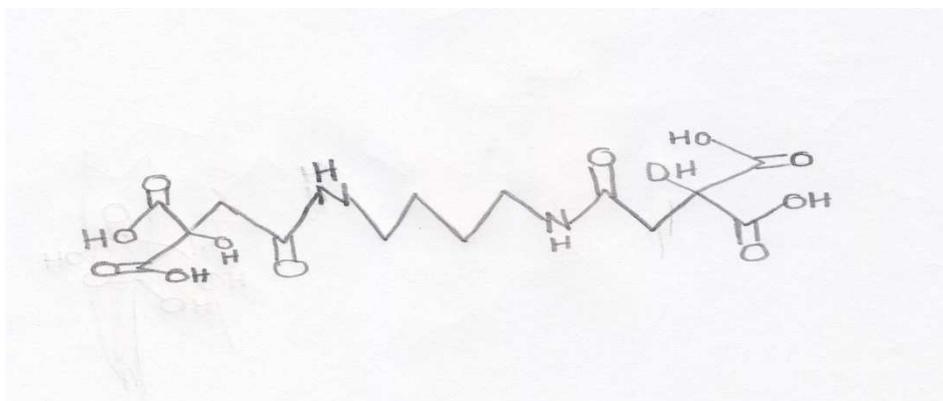
Figure 9. Rhizoferrin. The figure shows the chemical structure of rhizoferrin.

Siderophores in the Chemotaxonomy of Microorganisms

Research has been done and report has been made on the use of siderophores as the basis to discriminate between saprophytic fluorescent pseudomonas species, fluorescent *Pseudomonas syringae,* and *Pseudomonas viridiflava* strains. And to distinguish between the two siderovars in *Pseudomonas syringae* pv. Aptata (Bultreys et al,. 2003). Evidence of the classification of pseudomonas aeruginosa isolates into three different siderophore types has been reported (J. Meyer, 2000).

Siderophores in the Chemotaxonomy of Fungi

Morphological features have been used most often, as basis for classification in most fungi, but the absence of sporulating stages and similarities in morphology, in a number of fungal species causes problems in identification and classification. Chemical methods such as chemotaxonomy are now being used to complement already existing taxonomic methods that uses morphology as basis for classification. Chemotaxonomy makes use of biomarkers or signatory metabolites whose presence or absence is use as basis of classification, some of these signatory metabolites include carbohydrates, fatty acids, amino acids and carboxylic acids with the most notable being α-α-trehalose, Palmitic acid, Glycine and Phenylacetic acid (Aliferis et al., 2011). To the best of my knowledge there are no siderophore based studies conducted on fungi with respect to taxonomy.

Siderophores in the Chemotaxonomy of *Rhizoctonia solani* Species Complex

The classification of *Rhizoctonia solani* species complex is traditionally done by hyphal anastomosis testing and molecular methods (Cubeta and Vilgalys, 1997), molecular methods using ribosomal DNA sequence analysis of *internal transcribed spacer* (Sharon et al., 2006),

DNA Bar-coding and chemotaxonomy based on metabolite profiling (Allen et al., 2003; Larsen et al., 2005; Frisvad et al., 2008) have currently been employed. To the best of my knowledge there are no siderophore based studies conducted on isolates in the *R. solani* complex with respect to taxonomy.

Siderophores and Pathogenicity of *Rhizoctonia solani* and Other Microorganisms

Research has shown that the pathogenicity of microorganisms is due to the presence of specific biomolecules produced by these organisms (Calderone and Fonzi, 2001), many biomolecules have been attributed to be responsible for the virulence of several microorganisms. Phenylacetic acid (PAA) is believed to be contributing factor to the pathogenicity of *Rhizoctonia solani*. Correlation analyses suggest that, isolates of *Rhizoctonia solani* that produce high PAA and its derivatives caused high mortality on tomato seedlings (Bartz et al., 2012). There have been attempts to study the relationship between siderophore production, as a determinant of virulence of some human parasitic bacteria.

The conclusion drawn from one of such studies was that siderophores form an integral part of this phenomenon, but further studies were required to confirm that they are the sole or major determinant of pathogenicity in those selected microbes (Pal and Gokarn, 2010). Another study showed that, siderophore biosynthesis by the highly lethal mould *Aspergillus fumigatus* is essential for virulence during mammalian *Aspergillus* infection (Schrettl et al., 2007). Another study identified and characterized the gene *sidA* of *Aspergillus fumigatus* to be necessary for virulence of this fungus. The gene *sidA* encodes l-ornithine N^5-oxygenase, the first committed step in hydroxamate siderophore biosynthesis (Hissen et al., 2005).

Work done on the ferroxidation/permeation iron Uptake system in the smut fungus *Ustilago maydis* showed that two siderophore auxotroph genes *sid1* and *sid2* contribute to the high affinity iron uptake system that is responsible for the virulence of the fungus (Eichhorn et al., 2006).

It has also been shown that a nonribosomal peptide synthetase (NPS6) responsible for the biosynthesis of extracellular siderophores by *Cochliobolus heterostrophus* (a maize pathogen)*, Fusarium graminearum* (a wheat pathogen)*, and Alternaria brassicicola* (a pathogen of *Arabidopsis thaliana*) is a virulence determinant and it is also involved in the tolerance of H_2O_2 (Oide et al., 2006).

CHAPTER 3

MATERIALS AND METHODS

The materials and methods were adapted from Bharathan et al. (2005) and Schwyn &

Neilands (1987).

Materials

Five isolates of fungi belonging to the *Rhizoctonia solani s*pecies were obtained from the

laboratory of Dr. N. Bharathan in the Department of Biology, Indiana university of

Pennsylvania. The isolates include, EGR4, 386, 357, TOM 7 and RS29.3. These isolates were

selected based on their respective abilities to produce variable metabolic footprints and

variability in virulence. Membrane filters (Tisch scientific, SF15098), Malt extract broth (Difco,

0264006), Potato dextrose agar (Difco, 9040669), PrepSep Extraction columns (fisher scientific,

P452), Chrome Azurol S (Acros Organics, 190050250), Acetonitrile (Fisher Chemical, A9965K-

4), Methanol (Fisher Chemical, A411-4).

Reagents

Chrome Azurol S (CAS) assay solution, which consist of the following solutions: (i) 2

mM Chrome Azurol S (CAS) stock solution: 0.121 g CAS in 100 ml distilled water, (ii) 1 mM

Iron (Fe) stock solution: 1 mM $Fecl_3.6H_2O$: 270.3 mg in 1000 ml distilled water, in 10 mM HCL

(iii) MES Buffer (substituted for Piperazine buffer as modified by Alexander and Zuberer, 1990).

MES (2-(N-morpholino) ethane sulfuric acid buffer: 9.76 g of MES in 50 ml water, the pH was

adjusted to 5.6 with 50% Potassium Hydroxide (KOH), (iv) Hexadecyltrimethylammonium

(HDTMA): 0.0219 g HDTMA in 50 ml distilled water, (v) 0.2 M 5-Sulfosalicylic acid: 0.051 g

in I ml distilled water (shuttle solution) stored in the dark.

Equipment

These include: Ultra-centrifuge (Beckman Coulter optima ultra-centrifuge LE-80K, Beckman Coulter, MN. USA), Biological safety cabinet (Nuaire Class II Type B2, NUAIRE, MN. USA), Autoclave (Amsco lab 250, Boston laboratory equipments, MA. USA), High performance liquid chromatography (Shimadzu HPLC, LC-2010HT, Shimadzu, MD. USA), Visiprep (SPE vacuum manifolds. 57031-U, Sigma Aldrich, MO, USA), UV-Visible Spectrophotometer (Hitachi U-3900, Hitachi, IL. USA).

Plate Transfers

This involves the transfer of isolates from the starter cultures to new plates, to ensure that pure cultures are obtained for the experiment. All glassware and tools to be used were autoclaved before the transfers were done. For 10 agar plates, 7.8 g of Potato Dextrose Agar (PDA) was weighed and dissolved in 200 ml of distilled water (20 ml per plate) in a 1 liter Erlenmeyer flask, the resulting mixture was then autoclaved for 30 mins. The flask was then allowed to cool under the biological safety hood with the UV light on, approximately 20 ml of the Potato dextrose Agar was poured into each of the 10 plates**,** the plates were allowed to cool until no condensation was present.

Using a sterile knife, small sections of each of the 5 starter cultures were transferred to the 10 plates, two plates for each isolate were made, each plate was labeled with the isolate's name, the date the starter culture was made, the date the new plate was formed and the initials of the experimenter, the plates were then wrapped in parafilm and stored upside down in a clean dry place.

Flask Transfers

After 6 days, the new plated isolates had grown into pure cultures and were ready to be transferred into flasks. All glassware and tools were autoclaved prior to the transfers, 30 g of malt extract broth was weighed into an Erlenmeyer flask containing 2 L of autoclaved water and mixed, approximately 300 ml of the mixture was poured into each of six flasks, a cotton ball was put at the top of each flask as a stop and an aluminum foil was wrapped around it. The 6 flasks and their contents were autoclaved for 30 mins, the flasks were then allowed to cool in the biological safety hood with the UV light on, with a sterile knife each of the newly plated 5 isolates was transferred into a flask, a label was placed on each flask which has the isolates name, date formed, initials of the experimenter and the date the plate transfers were made.

An update of the flask inventory spreadsheet was made, the sixth flask served as a control containing only the culture media in order to discriminate between fungal derived metabolites and components of the media. Prior to the making of these 5 fungal cultures, 2 cultures were made, one each for TOM 7 and RS29.3, these served for the purpose of preliminary chromatographic investigations. They were chosen based on differences in their virulence and their abilities to produce different fungal metabolome.

Preliminary Sample Preparation for Liquid Chromatography

Changes in the color of the fungal cultures were observed after the first 7 days of growth. After 21 days of growth, approximately 50 ml of each of the two selected isolates, TOM 7 and RS29.3 were filtered on ice using membrane filters and centrifuged at 15,000 x g for 10 mins, at 4°C using the Beckman Coulter, optima ultra-centrifuge.

The supernatants were collected into clean disposable plastic tubes, capped and labeled. These metabolic footprints obtained were kept on ice for further analysis. These two isolates were selected for the preliminary investigations based on their varying abilities to produce metabolic footprints and their differences in virulence.

Solid Phase Extraction Using Disposable PrepSep Extraction Columns

Two columns were set up on the Visiprep SPE vacuum manifolds and cleaned by running 1 ml of methanol through them, this was followed by 1 ml of H_2O (milli Q). 3 ml of methanol was again run through the columns, followed by 3 ml of acetonitrile, the columns were then air dried under vacuum. After cleaning, 1 ml of acetonitrile was applied through each column, followed by 1 ml of H_2O (milli Q). 5 ml of the sample from each isolate was then applied to a labeled column, after the samples have run through the columns, 1 ml of H_2O (milli Q) was applied to rinse the columns, the eluates were collected. 1 ml of acetonitrile was then applied to the columns and the eluates again collected, finally 1 ml of H_2O (milli Q) was applied and the eluates were as well collected. In all 6 samples were collected for the preliminary liquid chromatography analysis. The collected eluates were then analyzed using the Shimadzu HPLC device.

Chrome Azurol S (CAS) Liquid Assay for the Detection of Siderophores

This method was developed by Schwyn and Neilands, and it uses an iron-dye complex which changes color on loss of iron, siderophores which have higher affinity for the iron than the dye can remove the iron, resulting in a change in color of the dye from blue to orange.

The Chrome Azurol S Assay was prepared as adopted from Schwyn and Neilands, and it comprises of the following solutions:

(i) 2 mM Chrome Azurol S (CAS) stock solution: 0.121 g CAS in 100 ml distilled water, (ii) 1 mM Iron (Fe) stock solution: 1 mM $FeCl_3.6H_2O$: 270.3 mg in 1000 ml distilled water, in 10 mM HCL, (iii) MES Buffer (substituted for Piperazine buffer as modified by Alexander and Zuberer, 1990). MES (2-(N-morpholino) ethane sulfuric acid buffer: 9.76 g of MES in 50 ml water, the pH was adjusted to 5.6 with 50% KOH , (iv) Hexadecyltrimethylammonium (HDTMA): 0.0219 g HDTMA in 50 ml distilled water, (v) 0.2 M 5-Sulfosalicylic acid: 0.051 g in I ml distilled water (shuttle solution) stored in the dark.

Procedure

After 28 days of growth, approximately 50 ml of each of the five selected isolates, TOM 7, RS29.3, 386, 357, EGR4 and the control were filtered on ice, using membrane filters, and centrifuged at 15,000 x g for 10 minutes, at 4ºC using the Beckman Coulter optima ultra-centrifuge. The supernatants were collected into clean disposable plastic tubes, capped and labeled. These metabolic footprints obtained were kept on ice. Six clean test tubes were labeled with the names of the 5 isolates and the control, 2 ml each of the culture supernatants and the control were pipetted into the labeled test tubes.

 2 ml of the chrome Azurol S Assay solution was added to each of the 6 test tubes, the mixture was then mixed thoroughly, 20 ul of shuttle solution was added to each complex and vortexed, the resulting dye complex was allowed to stand for 10 mins. Siderophores if present will remove iron from the dye complex resulting in a color change from blue to orange.

The mean absorbance of each of the five samples from the 5 fungal isolates and the control were measured at 630 nm for loss of blue color using the Hitachi U-3900 spectrophotometer .The relative percentage siderophore units were calculated from the measured absorbance. A four-week time-course study was performed to justify the results obtained from the experiment.

Time –Course Study for Siderophore Production

The materials and methods were adapted from Bharathan et al. (2005) and Schwyn & Neilands (1987). All protocol were followed as described in Chapter 3 (Materials and Methods) to make 5 fungal cultures for the selected isolates namely, TOM 7, EGR4, 386, 357, and RS29.3. A sixth flask serving as control, containing only the culture media was made in order to discriminate between fungal derived metabolites and components of the media. The Chrome Azurol S liquid Assay protocol was followed to prepare samples for spectrophotometric analysis, for the determination of siderophore production. A 4 week time-course study was performed, the mean absorbance of the 5 samples from the 5 fungal isolates and the control were measured at 630 nm, for the following periods, 7 days, 14 days, 21 days, and 28 days. The relative percentage siderophore units were calculated from the measured absorbance, the results obtained were used to generate a time –course curve to show the pattern of siderophore production across the 5 isolates with respect to time.

CHAPTER 4

RESULTS

High Performance Liquid Chromatography

Results from the High Performance Liquid Chromatography (HPLC), showed that TOM

7 has a peak with a retention time of 6.726 mins in the acetonitrile eluate, and 6.647 mins in the

water eluate, the peak indicate the presence of a compound in the sample and the retention time

indicates how long it takes for the compound to come out of the HPLC after injection. Retention

times are means of qualitative identification of compounds.
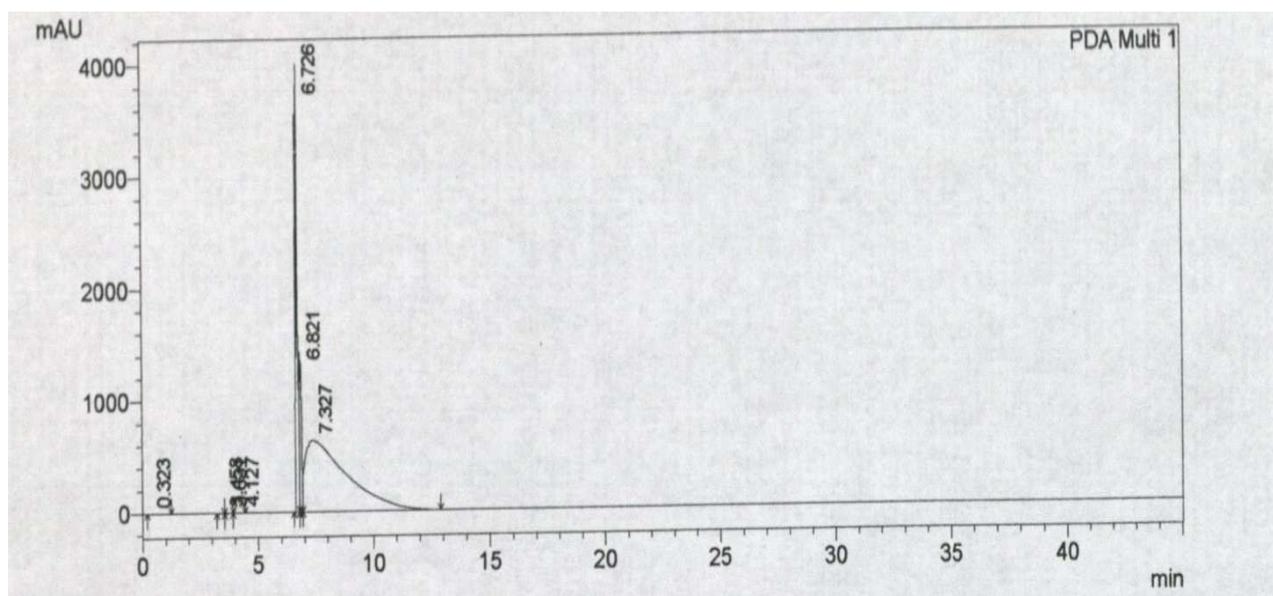
Figure 10. Chromatogram of TOM 7 in acetonitrile eluate. The figure shows the sharp peak and unique retention time of the isolate, TOM 7 in acetonitrile eluate.
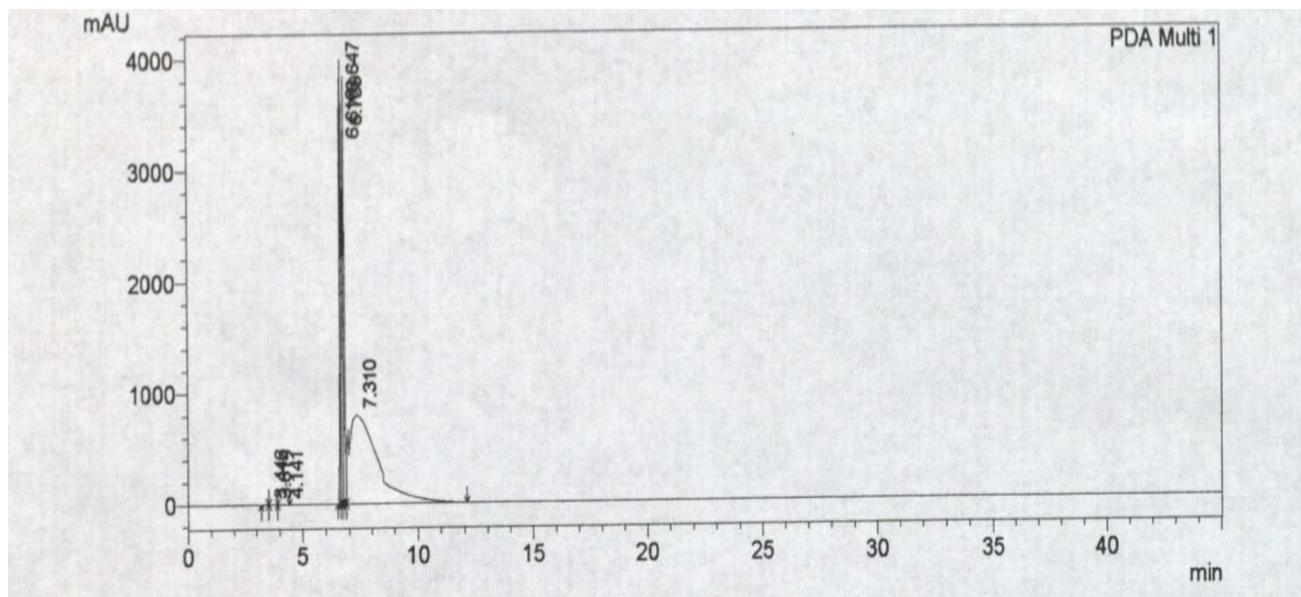
Figure 11. Chromatogram of TOM 7 in water eluate. The figure shows the sharp peak and unique retention time of the isolate, TOM 7 in water eluate.

The chromatogram for RS29.3 showed peak with retention times of 7.455 mins in acetonitrile eluate and 7.433 mins in water eluate, this also indicates the presence of a compound. These results gave hope that the selected *Rhizoctonia solani* isolates have the capacity to produce metabolic footprints needed for metabolic profiling.
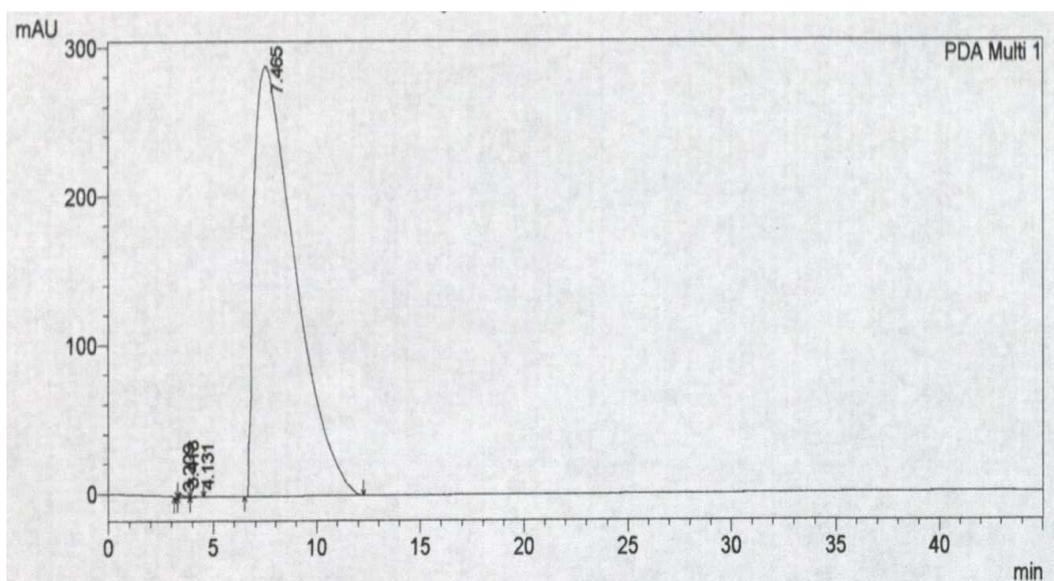
Figure 12. Chromatogram of RS29.3 in acetonitrile eluate. The figure shows the sharp peak and unique retention time of the isolate, RS29.3 in acetonitrile eluate.
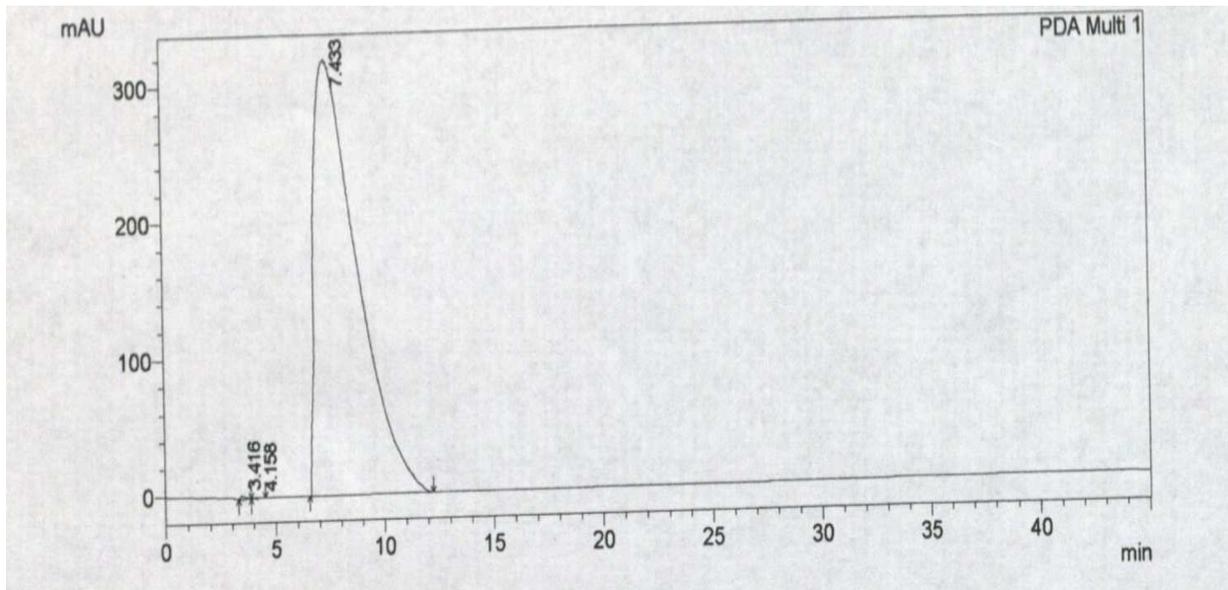
Figure 13. Chromatogram of RS29.3 in water eluate. The figure shows the sharp peak and unique retention time of the isolate, RS29.3 in water eluate.

Chrome Azurol S (CAS) Liquid Assay

Relative color change from blue to orange was observed across the 5 fungal cultures,

indicating the possible presence of Siderophores in the selected *R. solani* species.
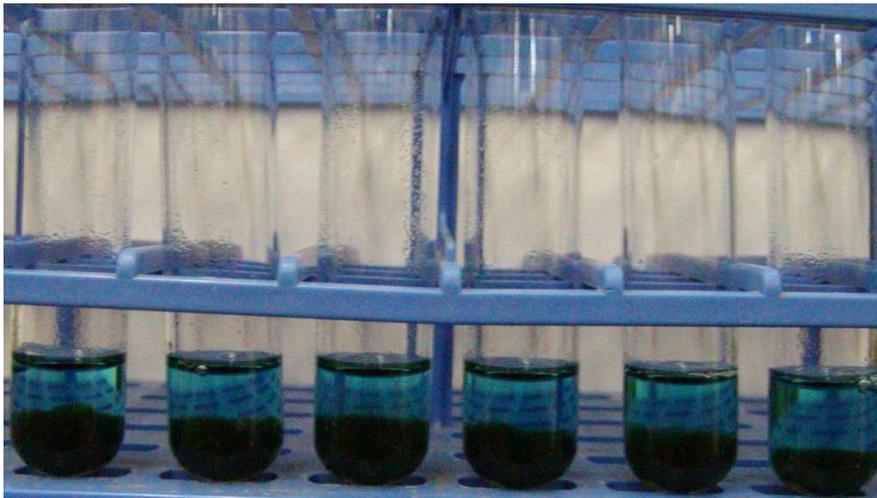
Figure 14. CAS assay solution only. The figure shows the blue color of the CAS assay solution without any culture supernatant.

Figure 15. CAS assay solution and culture supernatant. This figure shows the relative change in color from blue to orange across the 5 fungal isolates of *R. solani,* upon the addition of culture supernatant to the CAS assay solution.

Siderophores competitively solubilize and take up Iron from solution, the color change from blue to orange represent loss of Iron molecules from the CAS Assay Solution, to the Siderophores present in the metabolic footprints of the Isolates. Hence Siderophores if present will result in samples showing relatively lower absorbance readings depending on relative siderophore concentrations in the samples.

Hence the higher the amount of siderophores present in a culture supernatant, the higher the amount of iron molecules that would be taken from the CAS assay solution, and the lower the absorbance of the resulting assay solution complex. The more intense the resulting orange color, the higher the amount of siderophore present in the culture supernatant, conversely the less intense the resulting orange color, the lower the amount of siderophore present in the culture supernatant.

Table 2

Relative Absorbance of the 5 Rhizoctonia solani Isolates.

Sample	Absorbance (630 nm)
Control only	0.009
Control + CAS Assay Solution	0.697
CAS Assay Solution only	0.745
386 + CAS Assay Solution	0.530
TOM 7 + CAS Assay Solution	0.378
EGR4 + CAS Assay Solution	0.565
357 + CAS Assay Solution	0.471
RS29.3 + CAS Assay Solution	0.622

Table 3

Percentage Siderophore Units of the 5 Rhizoctonia solani Isolates.

Isolate	Absorbance (630 nm)	Percentage Siderophore Units (%)
386	0.530	28.9
TOM 7	0.378	49.2
EGR4	0.565	24.2
357	0.471	36.8
RS29.3	0.622	16.5

Percentage Siderophore units = [(absorbance of reference solution – absorbance of sample)/ absorbance of reference solution] ×100. Absorbance of reference solution (CAS Assay solution) = 0.745.

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Time –Course Study for Siderophore Production

The absorbance for each isolate over the 4 week period decline periodically over time, possibly indicating an increase in siderophore production by the isolates with time.

Table 4

Absorbance of the 5 Rhizoctonia solani Isolates Over the 4 Week Period.

Time (days)	TOM 7	357	386	EGR4	RS29.3
7	0.432	0.488	0.585	0.588	0.699
14	0.410	0.445	0.569	0.584	0.659
21	0.391	0.405	0.546	0.575	0.626
28	0.378	0.398	0.493	0.562	0.602

Table 5

Percentage Siderophore Units of the 5 Rhizoctonia solani Isolates Over the 4 Week Period.

Time (days)	TOM 7	357	386	EGR4	RS29.3
7	41.9	34.4	21.3	21.0	6.1
14	44.7	40.0	28.3	21.3	11.2
21	47.0	45.1	24.7	22.1	15.2
28	48.7	46.0	33.1	23.7	17.9

Percentage Siderophore units = [(absorbance of reference solution – absorbance of sample)/ absorbance of reference solution] ×100. Absorbance of reference solution (CAS Assay solution) = 0.744, 0.742, 0.738, and 0.737 for the first to the fourth week respectively.
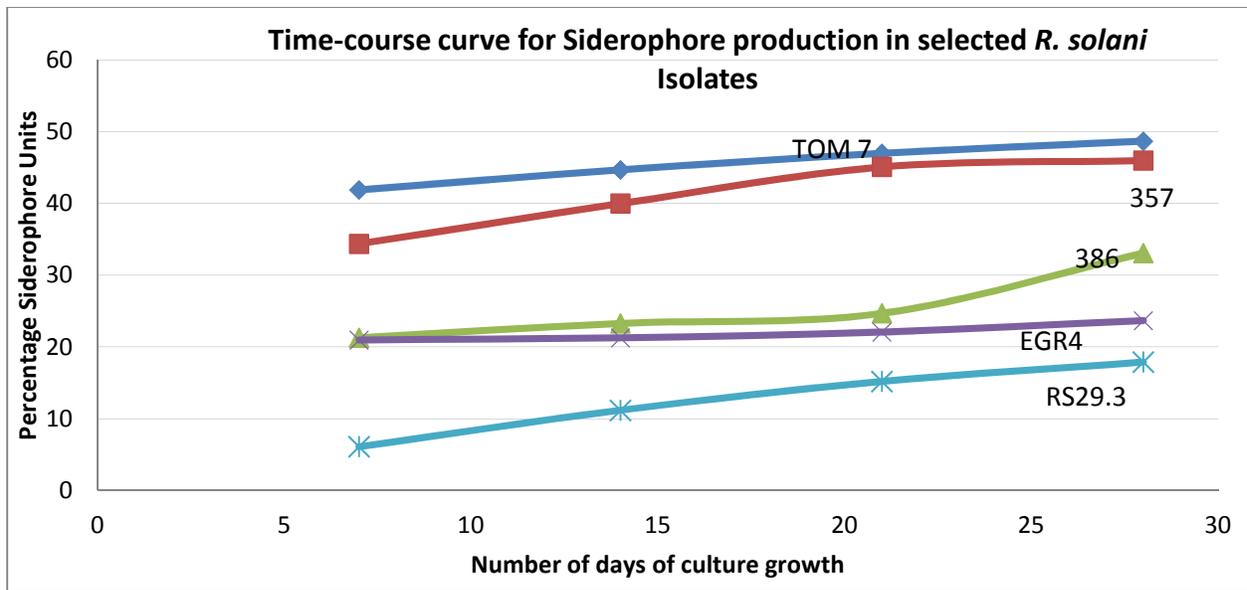
Figure 15. Time-Course Curve for Siderophore production in the 5 isolates. The graph shows the pattern of siderophore production with time, during the 4-week period in the 5 fungal isolates.

CHAPTER 5

DISCUSSION

Siderophores and the Pathogenicity of *R. solani*

Rhizoctonia solani causes diseases on a lot of plants including cotton, tobacco, tomato and potato, it attacks its hosts in their juvenile stages of development which are typically found in the soil, and hence it is considered as a soil-borne pathogen (A. Ogoshi, 1987). The fungus is found in the upper layers of the soil and can spread rapidly at temperatures below 68°F, the pathogen prefers warm wet weather, and outbreaks usually occur in the early months of summer. It can survive in the soil for many years in the form of sclerotia, these sclerotia can travel by means of wind, water or soil movement between host plants (Cubeta and Vilgalys, 1997). The fungus is attracted to the host plants by chemical stimuli released by a growing host plant and/or decaying plant matter. The most common symptom of *Rhizoctonia solani* disease is called "dumping of " which is characterized by non germination of affected seeds, other symptoms include seed decay, stem cankers, fruit decay and foliage diseases (Cubeta and Vilgalys, 1997), some of these consequences are major yield losses and poor industrial quality of the crops.

The pathogenicity of most fungi is believed to be due to the presence of specific biomolecules produced by these organisms (Calderone and Fonzi, 2001), phenylacetic acid (PAA) is believed to be a contributing factor to the pathogenicity of *Rhizoctonia solani,* this is supported by a correlation analysis that, isolates of *Rhizoctonia solani* that produce high PAA and its derivatives caused high mortality on tomato seedlings (Bartz et al., 2012).

This study, to the best of my knowledge could be the first attempt, or one of the pioneering efforts to find out if these selected isolates of *Rhizoctonia solani* produce siderophores, to quantify the amount of siderophore produced by each selected isolate, and to determine if there is a correlation between the degree of virulence and the amount of siderophore produced. Under iron-deficiency conditions, many heterotrophic bacteria and fungi have been reported to biosynthesize siderophores for their iron uptake (Reid and Butler, 1991; Muyzer et al 1993; Meyer et al., 1997) especially in marine bacteria, pseudomonas, and fish pathogens. In all such cases siderophore production is thought to occur commonly in due to the low iron concentration. In this study, we investigated siderophore production by *R. solani* fungi isolated from both field and laboratory manipulated cultures, which had reduced genome complement. We have compared the iron content of five different cultures and reported the detection of siderophore production.

High Performance Liquid Chromatography

Results from the preliminary HPLC studies showed that the two isolates TOM 7 and RS29.3 both had sharp peaks and unique retention times, figures 10 and 11 respectively show that TOM 7 had peaks with retention times of 6.726 mins in the acetonitrile eluate, and 6.647 mins in the water eluate. Figures 12 and 13 respectively show that RS29.3 had peaks with retention times of 7.455 mins in acetonitrile eluate and 7.433 mins in water eluate. These sharp peaks and unique retention times are qualitative indication of the presence of unique compounds. Further investigations are however needed to ascertain the identity of these compounds.

Chrome Azurol S Liquid Assay

From the CAS liquid screening assay, relative color change from blue to orange was observed across the 5 fungal cultures as shown in figures 13a and 13b, indicating the possible presence of siderophores in the selected *R. solani* species. Siderophores competitively solubilize and take up iron from solution, the color change from blue to orange represent loss of iron molecules from the CAS assay Solution, to the siderophores present in the metabolic footprints of the isolates (Schwyn and Neilands, 1987).

This resulted in samples showing relatively lower absorbance readings as compared to the CAS assay solution only, as shown in table 2, the difference in the absorbance of the 5 samples from the isolates depends on the relative siderophore concentrations in each sample, the lower the absorbance of the sample, the higher its siderophore concentration as shown in table 3.

The results obtained show that the 5 isolates selected for the experiment produce relative amounts of Siderophores which can be qualitatively identified and also quantified. This study agrees with literature which has TOM 7 to be the most virulent of the 5 isolates (Bartz et al., 2012) while RS29.3 which was engineered in the lab of Dr. N. Bharathan and it is still undergoing investigations, is the least virulent. In general, it was observed that, the amount of siderophore units that is produced is directly proportional to the virulence of the selected isolates, this indicates that the amount of siderophore produced by an isolate is a determining factor in its virulence. The growth stimulation of some terrestrial bacteria has been reported by the addition of exogenous siderophores (Champomier et al., 1996; Crosa J. H, 1997).

It is not clear whether those bacteria might have been stimulated to synthesize their own siderophores unlike what we see in *R. solani* in the present study. Studies on iron metabolism in terrestrial and pathogenic strains have shown that bacteria have established a variety of mechanisms by which to acquire chelated iron.

Time-Course Study

From the time-course study, it was observed as shown from table 4, that the absorbance for each isolate over the 4 week period decline periodically over time, indicating an increase in siderophore production by the isolates with time as shown in table 5. The field of metabolomics deals with the study of chemical fingerprints associated with specific cellular processes, these chemical fingerprints are a result of translation of genetic information. Currently, metabolomics is being used to complement traditional methods used in taxonomy, pathology and ecology, in these cases a number of metabolites are being adopted as biomarkers which are reliable indicators of these phenomena.

In conclusion, this study has shown that siderophores are potential determinants in the virulence of the selected isolates of *Rhizoctonia solani* species complex, the amount of siderophore produced is directly proportional to the level of virulence of these isolates. The way forward for this study is to involve a higher number of isolates of *Rhizoctonia solani,* as well as expand the study to other closely related species, and to chemically identify the type of siderophores being produced by these isolates and species, after which I will push for the adoption of these siderophores as biomarkers in the taxonomy, pathology and ecology of *Rhizoctonia solani* species complex.

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