IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA* MUTANTS UNABLE TO CONVERT RICINOLEIC ACID INTO 7,10,12-TRIHYDROXY-8(E)-OCTADECENOIC ACID (TOD) AND A SURVEY OF THE BIOLOGICAL ACTIVITY OF TOD

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IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA* MUTANTS UNABLE TO CONVERT RICINOLEIC ACID INTO 7,10,12-TRIHYDROXY-8(E)-OCTADECENOIC ACID (TOD) AND A SURVEY OF THE BIOLOGICAL ACTIVITY OF TOD

Taylor Boozer Hatchett

A Thesis

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Master of Science

Auburn, Alabama
May 9, 2009

IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA* MUTANTS UNABLE TO

CONVERT RICINOLEIC ACID INTO 7,10,12-TRIHYDROXY-8(E)-

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BIOLOGICAL ACTIVITY OF TOD

Taylor Boozer Hatchett

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VITA

Taylor Boozer Hatchett, daughter of Bobby and Sonya Boozer was born on 13 February 1984 in Auburn, Alabama. She graduated from Chilton Christian Academy in Jemison, Alabama, in 2002. Mrs. Hatchett received a Bachelor of Science degree in Agronomy and Soils from Auburn University, Auburn, Alabama, in 2005. Upon completion of her B.S. Mrs. Hatchett entered the Graduate School, Auburn University, Alabama, in January 2006, where she began work towards a Master of Science degree in Plant Pathology. Mrs. Hatchett was married on the 27 May 2006 to Jeremy Matthew Hatchett and the couple currently resides in Calera, Alabama.

THESIS ABSTRACT

IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA* MUTANTS UNABLE TO CONVERT RICINOLEIC ACID INTO 7,10,12-TRIHYDROXY-8(E)-OCTADECENOIC ACID (TOD) AND A SURVEY OF THE BIOLOGICAL ACTIVITY OF TOD

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Master of Science, May 9, 2009
(B.S., Auburn University, Auburn 2005)

82 Typed Pages

Directed by Kathy S. Lawrence

Rice blast, caused by *Magnaporthe grisea*, is a devastating problem in all rice producing areas of the world and is extremely difficult to prevent and/or to control. Therefore, it is critical to develop new methods to combat *M. grisea* which is estimated to cause the loss of enough rice to feed 60 million people each year.

The gram-negative bacterium *Pseudomonas aeruginosa* has the ability to convert ricinoleic acid into 7,10,12-trihydroxy-8(E)-octadecenoic acid (TOD). Kuo et al. (2001) found TOD to have antifungal activity against *Magnaporthe grisea*, *Monilinia fructicola*, *Phytophthora infestans*, and *Rhizoctonia solani*. The long-term goal of this research

project is to develop an efficient process for improving production of TOD from *P. aeruginosa* to mass-produce an economically competitive and environmentally friendly biological crop protection agent.

As an initial step towards this goal, a genetic study was undertaken to identify the genes that are required for production of TOD in *P. aeruginosa*. Using transposon mutagenesis, Jessica Cofield isolated 15,168 independent insertion mutants. From initial genetic screens, 107 insertion mutants that appeared to be unable to convert ricinoleic acid into TOD were identified.

In addition, a study was undertaken to assess the antimicrobial activity of TOD. Assays using 96 and 12-well microtiter plates were developed and utilized for the in vitro screening of 23 species of fungi, 17 species of bacteria, and 2 species of nematodes for sensitivity toward TOD. In the 96-well microtiter plate assay, *M. grisea's* growth was inhibited by TOD at 150μg/ml. The additional 22 species of fungi screened were not inhibited by TOD at this concentration. Of the 17 bacterial species screened, growth of 15 were significantly inhibited by TOD compared to the ethanol control. The average zone of inhibition ranged from 1.50 cm to 2.01cm. Mobility and morphological development of the plant pathogenic nematodes *Rotylenchulus reniformis* and *Meloidogyne incognita* was not affected by TOD. The ability of these nematodes to infect cotton roots was also unaffected by TOD compared to the untreated control. Therefore, the antifungal activity of TOD appears to be specific against *M. grisea* although the data suggest a wider antibacterial activity. These data will be instrumental in assessing the efficacy of TOD in agricultural applications.

ACKNOWLEDGMENTS

I first want to thank God for all the blessings in my life and the opportunities He has allowed me to have. I would like to thank my husband Jeremy whose love and support have been invaluable resources to me as I have pursued my graduate degree. I also wish to express my deepest gratitude to my parents who sacrificed in order for me to achieve a higher education. They have taught me so much about life and I would not be anything if it was not for their love and devotion. I want to thank my sister Whitney for always being willing to help me whenever I was running low on energy or enthusiasm or simply needed to laugh. I want to thank Dr. Lawrence, Dr. Suh, and Dr. Morgan-Jones. Last but certainly not least I would like to thank Jessica Cofield for her major contributions to this research project. I am so glad to have had the opportunity to get to know her. WAR EAGLE!

Style manual or journal used: Biological Control

Computer software used: Microsoft Word 2003, Microsoft Excel 2003

TABLE OF CONTENTS

LIST OF TABLES x

LIST OF FIGURES xi

I. INTRODUCTION 1

II. IDENTIFICATION OF POTENTIAL *PSUEDOMONAS AERUGINOSA* MUTANTS UNABLE TO CONVERT RICINOLEIC ACID INTO 7,10,12-TRIHYDROXY-8(E)-OCTADECENOIC ACID
- Abstract 17
- Introduction 18
- Materials and Methods 21
- Results and Discussion 26
- Summary 29

III. SURVEY OF THE BIOLOGICAL ACTIVITY OF A NOVEL ANTIFUNGAL COMPOUND PRODUCED BY *PSEUDOMONAS AERUGINOSA*
- Abstract 36
- Introduction 37
- Materials and Methods 41
- Results and Discussion 46
- Summary 51

IV. LITERAURE CITED 62

LIST OF TABLES

1. Inhibitory concentration of TOD (µg/ml) against *Magnaporthe grisea* using the microtiter plate assay 33

2. *Magnaporthe grisea* mycelial growth as affected by supernatants from *Pseudomonas aeruginosa* strains NRRL B-800 (800) and NRRL B-23260 (23260) at increasing volumes 34

3. Plate designation of potential TOD$^-$ mutants identified 35

4. Fungal isolates screened, classification and common disease they cause 55

5. Bacteria genus species and classification codes of isolates screened 56

6. Effect of TOD on fungal isolates as measured by mycelium growth 57

7. Effect of TOD and ethanol (EtOH) on bacterial isolates' growth as measured by the zone of clearing 58

8. Nematicidal activity of TOD on *Rotylenchulus reniformis* (reniform) and *Meloidogyne incognita* (root-knot) after 2 h exposure in vitro 59

9. Effect of TOD on ability of *Rotylenchulus reniformis* and *Meloidogyne incognita* to colonize cotton roots 60

10. Effect of TOD on the development of *Rotylenchulus reniformis* and *Meloidogyne incognita* on cotton roots 61

LIST OF FIGURES

1. Diagram of a fatty acid showing hydroxyl groups..13

2. Examples of mono- di- and tri- hydroxy fatty acids..14

3. Comparison of (1) TOD and (2) a naturally produced trihydroxy fatty acid from a cultivar of rice resistant to rice blast...15

4. Schematic of proposed conversion pathway by which *Pseudomonas aeruginosa* PR3 converts ricinoleic acid into 7,10,12-trihydroxy-8(E)-octadecenoic acid 16

5. Schematic of proposed conversion pathway by with *Pseudomonas aeruginosa* PR3 converts ricinoleic acid into 7,10,12-trihydroxy-8(E)-octadecenoic acid 30

6. Bi-parental mating between *Escherichia coli* donor and *Pseudomonas aeruginosa* recipient...31

7. Diagram of mutant hunt..32

8. *Rhizobium radiobacter* 89C-02 from bacterial screen showing zone of inhibition by TOD 53

9. Fourteen day old cotton roots inoculated with *Meloidogyne incognita* and treated with TOD, ethanol, oxamyl, and aldicarb...54

I. INTRODUCTION

Rice blast, the most severe disease of cultivated rice (*Oryza sativa*), is a devastating disease plaguing much of the world's rice production (Sesma and Osbourn, 2004). Rice is the main food staple for more than one third of the world's population and it is estimated that world production will be 421 million tons in 2007/2008 (USDA, 2008).

Magnaporthe grisea (Hebert) Barr [anamorph *Pyricularia grisea* (Cooke) Sacc], the causal agent of rice blast disease is found worldwide. This pathogen is estimated to cause the loss of more than 150 million tons of rice or enough rice to feed 60 million people each year (Talbot, 2003; Zeigler et al., 1994). *Magnaporthe grisea* contains subgroups that cause diseases not only on rice, but also on foxtail, common, and finger millet, crabgrass, and wheat (Kato, 2001) and other diverse species of grasses and sedges (Shim et al., 2004).

The sexual stage of *M. grisea* rarely occurs in nature (Talbot, 2003). Rather, the fungus carries out asexual reproduction to produce three celled spores called conidia (Talbot, 2003). The conidia, which are spread by wind and rain, can attack rice at all growth stages from seedling through maturity and can infect and invade both leaves and stems (Bourett and Howard, 1990). The most devastating losses in rice production occur when the panicle or node at the base of the panicle is infected and killed, leading to a complete loss of grain set (Ou, 1985). In the southeastern United States of America,

warm temperatures and rainy spells between May and September cause high relative humidity and leaves often remain wet for periods of 12 hours and longer. These extended periods of leaf wetness combined with 80° to 90 °F temperatures, create ideal environmental conditions for *M. grisea* infection, growth, and colonization (Harmon et al., 2005). The conidia of *M. grisea* attach to leaves with an adhesive released from their tip (Hamer et al., 1988). In the presence of adequate moisture and temperature, *M. grisea* conidia can germinate in as little as two hours and form a germ tube (Hamer and Talbot, 1998). It is the germination of the conidia from *M. grisea* on the leaf surface that initiates the infection process (Dean et al., 2005). The germ tube grows for a short period of time before developing into an appressorium, which is a highly specialized, dome-shaped cell (Zeigler et al., 1994). During the formation of the appressorium the germ tube stops growing, new cell wall materials are synthesized, an adhesive is produced to bind the appressorium securely to the leaf surface and a specialized septum forms at the base (Hamer and Talbot, 1998). Once the appressorium forms, hyphal growth is able to begin again (Hamer and Talbot, 1998). As the appressorium develops, it expands and becomes melanized (Bourett and Howard, 1990). The zone at the base of the appressorium does not become melanized and this is where the penetration peg develops (Howard and Valent, 1996). The peg is able to penetrate the host cell wall allowing hyphae to develop within the leaf epidermis (Howard and Valent, 1996). Penetration is made possible by turgor pressure that builds up inside of the appressorium. The appressorium is capable of producing up to 8 MPa of turgor pressure (De Jong et al., 1997). The turgor pressure is generated through the accumulation of 3.22 M concentration of glycerol which the appressorium is capable of maintaining because of its polyketide melanin layer (De Jong

et al., 1997). A primary infection hypha forms as the peg enlarges after the cell wall of the plant is breached (Heath et al., 1990, 1992; Koga, 1994). The hypha quickly develops into secondary, branching hyphae and moves throughout the host cells (Heath et al., 1990, 1992; Koga, 1994). The hyphae enlarge to encompass both intra and extra cellular spaces (Hamer and Talbot, 1998). Three days after inoculation the fungal hyphae may make up to 10% of the total biomass in the host leaves (Talbot et al., 1993). A disease lesion is produced by 4 days after infection, conidiophores emerge from these lesions and the fungus sporulates spreading the disease to adjacent plants (Hamer and Talbot, 1998).

Historically rice blast has been controlled with the use of resistant cultivars and fungicides (Chao and Ellingboe, 1997). The use of resistant cultivars has been the preferred means of disease management because of the economical and environmental advantages they provide compared to the use of synthetic pesticides (Levy et al., 1993; Zeigler et al., 1995). However, *M. grisea* has a high level of genetic variability and thus there is always the potential for new host-specific forms to evolve (Dean et al., 2005; Consolo et al., 2005; Takabayashi et al., 2002; Zeigler et al., 1997). These variants are often able to infect formerly resistant host plants and have occurred frequently during rice cultivation (Bonman et. al, 1991). Unlike many plant pathogenic fungi, *M. grisea* strains in a population mutate from an avirulent form to a virulent form (Lao and Ellingboe, 1993). This shift from avirulent to virulent strains is frequently associated with the inactivation or deletion of genes that code for proteins that activate the plant's response to the pathogen (Dean et al., 2005; Farman, 2002; Kang et al., 2001). Because many of the host-specific genes in *M. grisea* are located in regions rich with transposons, their inactivation allows for an increase in host range and increase in virulence (Dean et al.,

2005; Farman et. al., 2002; Kang et. al., 2001). These continuous alterations in disease pathotype have greatly limited the use of resistant varieties (Shim et al., 2004; Uddin, 2000). For instance, in 1989 the rice cultivar Katy was released by Arkansas plant breeders (Moldenhauer et al., 1990). Katy carried the *Pi-ta* gene for blast resistance which was discovered in the wild-type rice, 'Tetep' (Moldenhauer et al., 1990). When first released, the *Pi-ta* gene conferred resistance to all blast strains commonly found in Arkansas production areas and additional cultivars were developed (Moldenhauer et al., 1990, 1998; Yulin et al., 2004). In 2004, however, the virulent strain IE-1K was detected on the cultivar 'Drew', which carried the *Pi-ta* gene (Lee et al., 2005). It is not know whether IE-1k evolved from within the native pathogen population as the result of natural selection pressure or from a spontaneous mutation for virulence. Bonman (1991) found that due to the great diversity of rice-growing environments, resistance that proves durable in one system may or may not prove to be useful in another.

Chemical treatment of rice blast is accomplished through the use of fungicides. Fungicides recommended for use in rice production in the United States to combat rice blast include azoxystrobin (Quadris 2.08 FL®), trifloxystrobin (GEM®), benomyl (Benlate®), propiconazole + trifloxystrobin (Stratego®), and azoxystrobin + propiconazole (Quilt®) (Cartwright et al., 2009; Ferrin et al., 2008). These fungicides are most effective when applied preventatively before the pathogen becomes active. Fungicide applications not only increase production costs, they may also cause a negative impact on soil microbial populations by inhibiting the growth of non-pathogenic, beneficial fungi (Elmholt and Smedegaard-Petersen, 1987). Elmholt and Smedegaard-Petersen (1987) studied the side-effects of field applications of propiconazol (Quilt®) and

captafol (Ortho Difolatan FW®) on the composition of soil fungi in spring barley. They found that significantly fewer fungi were isolated from barley plots treated with propiconazol or captafol than from untreated barely plots. The number of primary saprophytic fungi was actually reduced for more than a month when treated with captafol (Elmholt and Smedegaard-Petersen, 1987).

Cultural practices are an extremely important method to prevent and reduce rice blast incidence due to increasing limitations with the use of resistant cultivars and synthetic chemical fungicides. In some areas of the world, growers' burn fields containing dead and diseased plant materials which may otherwise serve as a source of inoculum for the following season's crop (Kato, 2001). However, burning is not viewed as a desirable, long-term control strategy due to environmental concerns from the air pollution caused by the smoke production (Delany et al., 1985). Plants are more likely to develop rice blast if they are stressed due to aerobic environmental conditions, excessive nitrogen fertilization, and drought stress (Krausz, 2005; Mukherjee et al., 2005). Cultural practices of reducing periods of drainage, combined with the application of proper rates of nitrogen, decrease but do not eliminate the threat of rice blast. Nitrogen recommendations vary and are made based on the yield potential of the particular cultivar of rice being grown, soil type, cultural practices, and crop rotations (Wilson et. al., 1998). Maintaining properly flooded conditions aids in reducing the threat of rice blast by eliminating drought stress and creating an anaerobic environment that is not conducive to the pathogen's lifecycle. Dry seeding, which is the standard management practice in the U.S., involves spreading the seeds onto the soil surface and then incorporating them into the soil (Helms and Slaton, 1994). This practice is discouraged in areas prone to rice

blast outbreaks because the fields are not flooded until the 5th leaf stage. Delayed flooding can enhance the pathogens' ability to be transmitted from the seed to the seedling (Helms and Slaton, 1994). To reduce pathogen transmission, seeds are germinated and spread onto flooded seedbeds (Helms and Slaton, 1994). Overall, although cultural practices can reduce the risk of disease infestation, they are not enough to control rice blast when disease pressure is high. New additional methods of disease management, including possible biological control agents, are needed.

Pyricularia grisea, the causal agent of gray leaf spot on perennial ryegrass turf, occurs throughout the United States and can cause severe turf losses (Correll et al., 2000; Farman, 2002). *Pyricularia grisea* is the anamorph state of *M. grisea* and the disease is named for the distinctive gray-centered leaf spots caused by this fungus (Harmon and Latin, 2003). The pathogen survives unfavorable environmental conditions in plant debris as dormant mycelium (Harmon and Latin, 2003). In the spring, the combination of frequent showers, high humidity and warm temperatures create favorable environmental conditions and the mycelium begins to grow and produce conidia which are dispersed by the wind (Hagan, 2000). In the southeast, gray leaf spot symptoms appear in late spring and continue into late fall (Elliott and Simone, 2008). The average golf course with turf susceptible to gray leaf spot can expect a 5% increase in their fungicide budget to manage the disease (Uddin et al., 2003). However, gray leaf spot is not considered a major threat to perennial ryegrass, which is typically planted over warm season turfgrasses and not as a primary turfgrass in this region (Horgan and Yelverton, 2001; Harmon and Latin, 2003). Gray leaf spot presents more danger to tall fescue (*Festuca arundinacea*) which is commonly grown in the southeastern region (Green et al., 1998). During the summer of

1999 in Alabama there was a serious outbreak of gray leaf spot on Rebel II fescue (Hagan, 2000).

Gray leaf spot symptoms, including leaf spots and leaf blights, are greatly influenced not only by the environmental conditions but also the age of the host (Harmon and Latin, 2003; Landschoot and Hoyland, 1992; Trevathan et al., 1994). Affected areas of perennial ryegrass and tall fescue may appear drought stressed. Irrigation, however, intensifies the problem and can result in more extensive disease development (Harmon and Latin, 2003). On immature perennial ryegrass (those less than 5 weeks old), leaf infections initially appear water-soaked and are misshapen and abnormal where the infection occurs (Landschoot and Hoyland, 1992; Trevathan et al., 1994; Williams et al., 2001). On tall fescue, gray leaf spot initially appears as round or oval tan colored spots with dark brown borders (Smiley et al., 2005). Disease outbreaks progress to leaf distortion, collapse, and plant death (Harmon and Latin, 2003). Immature stands (those less than 5 weeks old) of perennial ryegrass and tall fescue are more susceptible to gray leaf spot than mature stands (Trevathan et al., 1994).

Cultural practices such as proper mowing, fertilization, and irrigation reduce the likelihood of severe turf loss from gray leaf spot disease (Harmon et al., 2005). Although cultivars may vary in their level of sensitivity, there are no varieties of perennial ryegrass or tall fescue which are completely immune to the disease. Possible sources of resistance have been identified in annual ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*), St. Augustinegrass (*Stenotaphrum secundatum*) and tall fescue (*Festuca arundinacea*) (Tredway et al., 2003, Bonos et al., 2004). These “resistant” varieties have

the same limitations as "resistant" rice cultivars due to the high number of pathogenic variations of *P. grisea*.

Fungicides recommended for gray leaf spot management in home lawns include azoxystrobin (Heritage 50DG), propiconazole (Banner Maxx), and trifloxystrobin (Compass 50W) (Everest, 2008). Commercial recommendations include those as well as chlorothalonil (Daconil Ultrex), chlorothalonil + thiophanate-methyl (Spectro 90), fenarimol + chlorothalonil (Broadway 4.4F), fludioxonil (Medallion), pyraclostrobin (Insignia), triadimefon (Bayleton T/O), and trifloxystrobin + triadimefon (Armada 50W) (Everest, 2008). The strong reliance on fungicides as a line of defense against gray leaf spot poses problems since repeated fungicide applications can lead to the development of fungicide-resistant strains (Park et al., 2005). For instance, in August 2000 gray leaf spot was diagnosed in strobilurin-treated perennial ryegrass turf on golf courses in Lexington, KY, Champaign, IL, and Bloomington, IL (Kim et al., 2003; Vincelli and Dixon, 2002). With the use of DNA fingerprinting it was discovered that the mutations occurred in five genetic backgrounds indicating that the field resistance discovered with the strobilurin fungicides was the result of a small number of mutations (Kim et al., 2003).

Plant defense mechanisms are often researched for plant disease management techniques (Narusaka et al., 2006). Plants are known to produce hydroxyl fatty acids, which serve as important industrial materials (Hou and Forman, 2000; Kato et al., 1984). The hydroxyl group gives the fatty acid the chemical properties of higher viscosity and reactivity compared to other fatty acids (Figure 1) (Kim et al., 2002). Fatty acids are aliphatic monocarboxylic acids that are found in animal fats, vegetable fats, oils, and waxes. Saturated fatty acids do not contain any double bonds or functional groups

whereas unsaturated fatty acids contain one or more alkenyl functional group and double bonds. Unsaturated fatty acids also possess a broad spectrum of biological properties (Bajpai et al., 2004; Walters et al., 2004). Hydroxy fatty acids often act as plant self-defense substances (Kato et al., 1983, 1985, 1986; Masui et al., 1989). Many rice cultivars resistant to rice blast disease produce oxygenated unsaturated fatty acids which act as antifungal substances (Kato et al., 1984, 1985, 1986). Kato et al. (1983, 1984, 1986) isolated unsaturated trihydroxy C_{18} fatty acids from a susceptible cultivar of rice suffering from rice blast disease and discovered those fatty acids to have antifungal activity. Walters et al. (2004) conducted an in vitro test to study the antifungal activities of four fatty acids produced by plants. Linolenic acid and linoleic acid reduced the mycelial growth of *Rhizoctonia solani* (70%, 74%), *Pythium ultimum* (56%, 65%), *Pyrenophora avenae* (42%, 33%), and *Crinipellis perniciosa* (50%, 43%) (Walters et al., 2004). Oleic acid reduced the mycelia growth of *P. ultimum* (63%) and *C. perniciosa* (35%) while erucic acid showed no antifungal activity (Walters et al., 2004).

In addition to antifungal compounds produced by plants, many microorganisms also have antagonistic properties against plant pathogenic fungi through bioconversions of unsaturated fatty acids (Magnusson et al., 2003). Unsaturated fatty acids produced by microorganisms fall into one of three categories based on the number of hydroxyl groups they contain: 1) monohydroxy, 2) dihydroxy and 3) trihydroxy (Figure 2) (Hou 1996; Kim et al. 2002; Kuo et al. 2001). Molecular modifications of fatty acids often lead to value-added products for a variety of new industrial uses such as lubricants, soaps, and cosmetics (Kuo et al., 2001; Kuo and Nakamura, 2004). Recent research into bacterial oxidation of fatty acids has led to the discovery of several hydroxy fatty acids with

antimicrobial capabilities. Avis and Belanger (2000) tested the fatty acid cis-9-heptadecenoic (CHDA) acid produced by *Pseudozyma flocculosa* for antifungal capabilities against seven different fungi: *Botrytis cinerea*, *Cladosporium cucumerinum*, *Idriella bolleyi*, *Phytophthora infestans*, *Pseudozyma rugulosa*, *Pythium aphanidermatum,* and *Sphaerotheca fuliginea*. CHDA exhibited activity against all of the fungi tested, although the degree of sensitivity varied considerably (Avis and Belanger, 2000). Mundt et al. (2003) studied the antibacterial activity of two unsaturated fatty acids, 9-hydroxy-10E,12Z-octadecadiencoic acid and 13-hydroxy-9Z,11E-octadecadiencoic acid isolated from cyanobacteria. In the agar plate diffusion test the two fatty acids inhibited the growth of the bacterial isolates *Bacillus subtilis* SBUG 14, *Micrococcus flavus* SBUG 16, *Staphylococcus aureus* SBUG 11 and *S. aureus* ATCC 25923 (Mundt et al., 2003). No activity, however, was observed against multi resistant *S. aureus* strains (Mundt et al., 2003).

Pseudomonas aeruginosa, a common soil bacterium has a large and complex genome that permits it to thrive in many different environments (Stover et al., 2000). This bacterium has the ability to convert exogenous ricinoleic acid (RA) into 7,10,12-trihydroxy-8(E)-octadecenoic acid (TOD) (Kim, H. et al., 2002). TOD closely resembles naturally occurring trihydroxy unsaturated fatty acids such as those produced by rice blast resistant cultivars (Kato et al., 1985, 1986). For example, the naturally occurring compound 9,12,13,-trihydroxy-10(E)-octadecenoic acid from rice plants is structurally similar to TOD and is known to possess antifungal qualities (Figure 3) (Kato et al., 1985, 1986; Kuo et al., 1998). Kuo et al., (2001) found TOD to have antifungal activity against *Monilinia fructicola*, *P. infestans*, *R. solani*, and *M. grisea*, the causal organisms,

respectively, of peach blossom blight, potato late blight, rice sheath blight and rice blast. To test TOD's antifungal activity, Kuo et al. (2001) sprayed each test plant with 15 ml of 5 parts per million of TOD in a mixture of acetone:water. After 24 h the plants were inoculated with the spores of the fungal pathogen being tested and placed in a growth chamber for 4 to 5 days (Kuo et al., 2001). The greatest percent of growth inhibition was reported with *M. grisea*, the causal agent of rice blast, at 29% (Kuo et al., 2001). Although 100% growth inhibition was not achieved, the growth of *R. solani*, *M. fructicola*, and *P. infestans* were inhibited21%, 20%, and 1% respectively (Kuo et al., 2001). The causal organisms of wheat foot rot (*Fusarium* spp.) and wheat glume blotch (*Septoria* spp.) exhibited no growth inhibition by TOD (Kuo et al., 2001).

The process of TOD purification outlined below was previously described by Kuo et al. (1998). Ricinoleic acid is a mono-hydroxy fatty acid derived from the castor plant and is added to 18 h old *P. aeruginosa* cultures grown in 30 ml of Wallen fermentation medium (Levinson et al., 2005). Maximum conversion of RA to TOD takes place between 36 and 48 h. However, even under ideal conditions only 40% of the RA is converted into TOD (Kuo et al., 2001). An increase in the amount of RA converted to TOD would make the process more efficient and serve as a very important step in the introduction of a biological fungicide.

Pseudomonas aeruginosa is an ideal bacterium to study because so much is already known about it genetically. The entire genome of 6.3 million nucleotides has been sequenced (Stover et al., 2000). Little is known, however, about the genes that are actually involved in the conversion process from RA to TOD, but it is hypothesized that

RA, an unsaturated fatty acid, goes through two hydroxylation steps before becoming a trihydroxy fatty acid (Figure 4) (Kim, H. et al., 2002).

The long-term goal of this collaborative project is to identify the genes involved in the conversion of RA into TOD and ultimately engineer a strain of *P. aeruginosa* capable of improved conversion. As an initial step toward achieving these goals, experiments were designed to screen random transposon insertion mutants of *P. aeruginosa* and identify those that are potentially defective in the conversion of RA to TOD. In addition to identifying *P. aeruginosa* mutants unable to convert RA to TOD, this research is also aimed at elucidating the antimicrobial range of TOD. Biological screens were conducted in order to test the in vitro effect of TOD on various species of fungi, bacteria, and nematodes.

Figure 1. Diagram of a fatty acid showing hydroxyl groups.

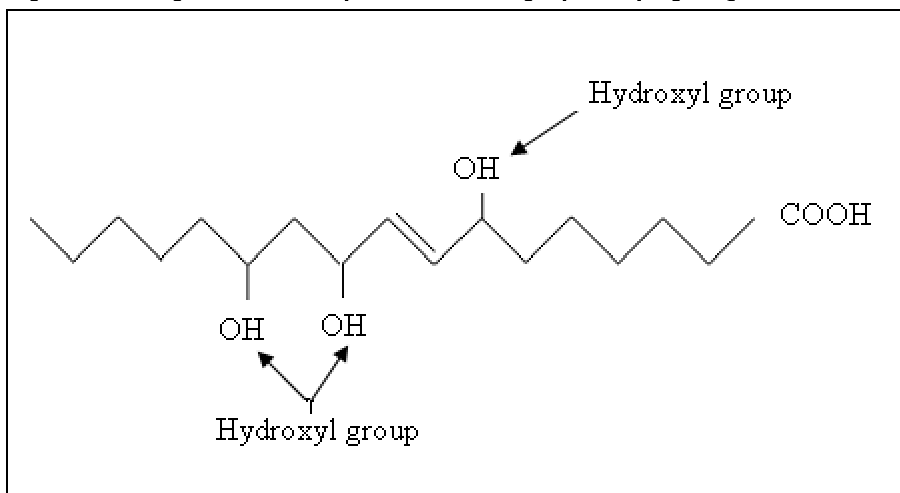


Figure 2. Examples of mono- di- and tri- hydroxy fatty acids.

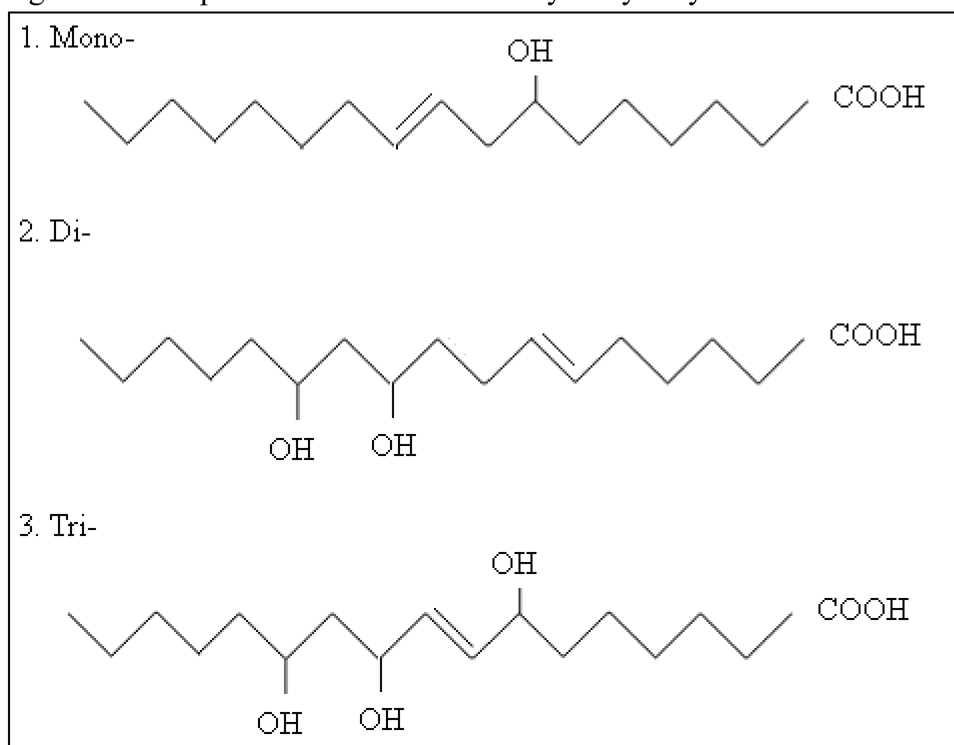


Figure 3. Comparison of (1) TOD and (2) a naturally produced tryihydroxy fatty acid from a cultivar of rice resistant to rice blast.

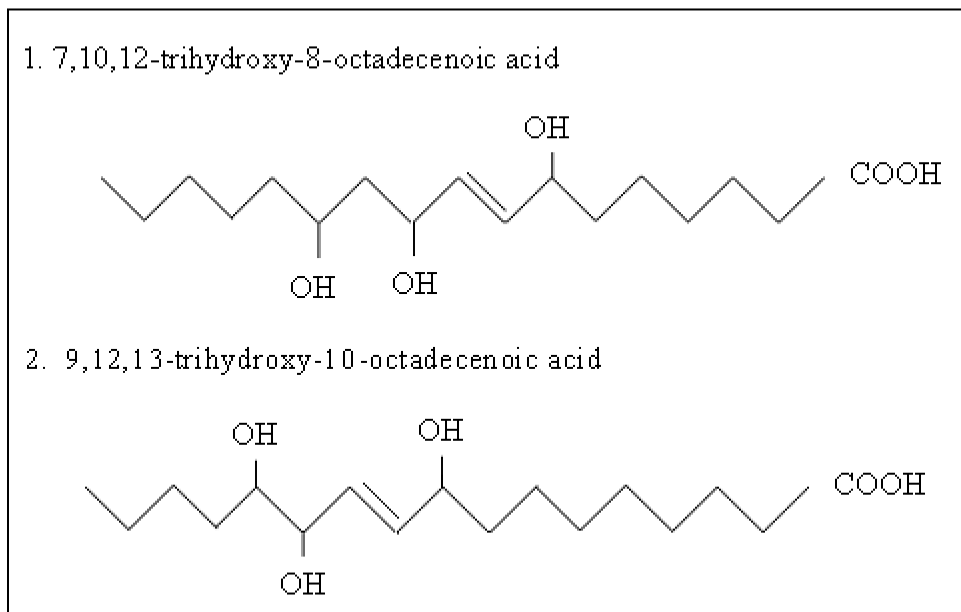
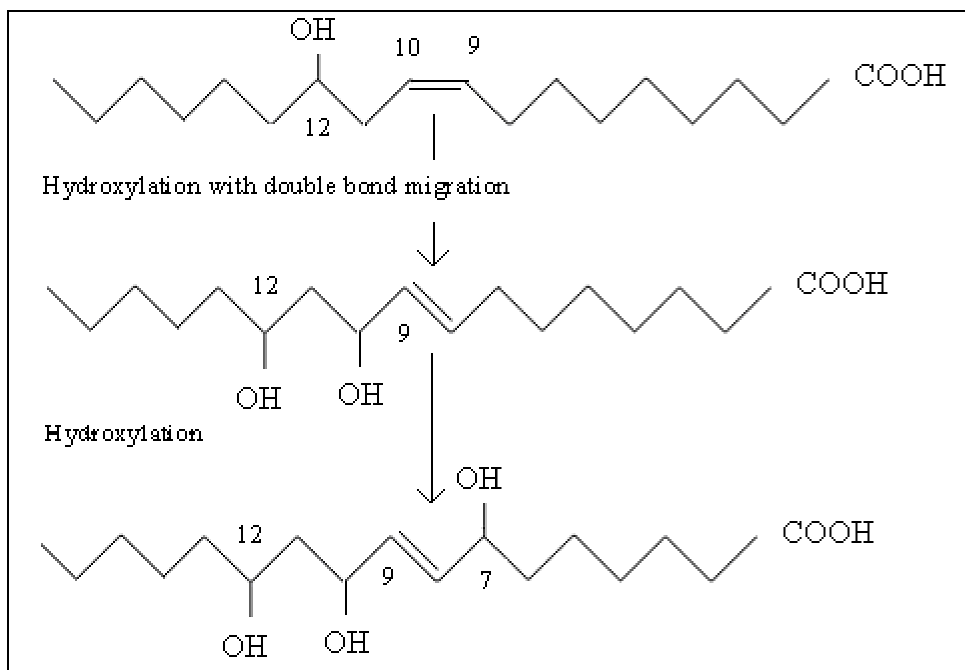


Figure 4. Schematic of proposed conversion pathway by which *Pseudomonas aeruginosa* PR3 converts ricinoleic acid into 7,10,12-trihydroxy-8(E)-octadecenoic acid.



I. IDENTIFICATION OF POTENTIAL *PSUEDOMONAS AERUGINOSA* MUTANTS UNABLE TO CONVERT RICINOLEIC ACID INTO 7,10,12-TRIHYDROXY-8(E)-OCTADECENOIC ACID

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Abstract

The gram-negative bacterium *Pseudomonas aeruginosa* catalyzes the conversion of ricinoleic acid (RA) into the novel trihydroxy fatty acid 7, 10, 12-trihydroxy-8(*E*)-octadecenoic acid (TOD) which has antifungal activity against *Magnaporthe grisea,* the causal agent of rice blast disease. Natural crop-protecting agents such as TOD offer several advantages over synthetic agents, including improved ecological compatibility and environmental safety. Unfortunately, because many naturally occurring crop-protecting agents are produced only in trace amounts, it has been difficult to isolate large enough quantities in an economically feasible manner. Thus, a bacterium such as *P. aeruginosa* that is genetically amenable and produces an antifungal agent is ideal for genetic manipulation to achieve improved TOD production.

The long-term goal of this research is to develop an efficient process for improving production of TOD from *P. aeruginosa* to mass-produce an economically competitive and environmentally friendly biological crop protection agent.

As an initial step towards this goal, a genetic study was undertaken to identify the genes that are required for production of TOD in *P. aeruginosa*. A bioassay was developed to assess TOD's antifungal activity against *M. grisea* in 96-well microtiter plates using either pure TOD or *P. aeruginosa* culture supernatant that contains TOD due to microbial conversion. Transposon mutants were then screened to identify those that are defective for TOD production. A library of 15,168 Tn*5*-B21 insertion mutants of the *P. aeruginosa* strain NRRL B-23260 (an environmental isolate) was screened.

Of the 15,168 insertion mutants screened for TOD production in the microtiter plate bioassay, 107 mutants appeared to be unable to convert RA to TOD upon initial screen.

1. Introduction

Worldwide rice is the number one food crop and provides the majority of the daily nutrients to more than half of the world's total population (Shim et al., 2004). A total of 406.1 billion tons of rice was produced in 2005/2006 (Shim et al., 2004). All plants are susceptible to diseases caused by various organisms and rice is no exception. Perhaps the most severe disease of cultivated rice (*Oryza sativa*) is rice blast, a devastating disease plaguing much of the world's rice production (Sesma and Osbourn, 2004). *Magnaporthe grisea*, the causal agent of rice blast disease is found worldwide and

is estimated to cause the loss of enough rice to feed 60 million people each year (Tani et al., 2005).

Historically, rice blast has been managed with the use of resistant cultivars and fungicides (Chao and Ellingboe, 1997). Resistant cultivars are an ideal means of preventing plant diseases because unlike synthetic chemical pesticides they do not cause a negative environmental impact. Unfortunately, *M. grisea* displays a high level of genetic variability maintaining a potential to evolve new virulent forms that are cultivar-specific (Takabayashi et al., 2002; Consolo et al., 2005; Zeigler et al., 1997). The strong reliance on fungicides as a line of defense against blast disease also poses problems (Park et al., 2005). Repeated fungicide applications can lead to the development of fungicide-resistant strains and have negative impact on non-target organisms (Viji et al., 2003). The negative environmental problems often associated with the use of synthetic compounds have made the development and utilization of natural crop-protecting antifungal agents more desirable.

Plants produce several trihydroxy unsaturated fatty acids that have antifungal activities (Kato et al., 1984, 1985, 1986, Masui et al., 1989). Unfortunately, plants produce these compounds only in minute quantities and therefore these molecules are difficult and costly to isolate. In the past, these difficulties have limited the utilization of natural antifungal compounds as potential crop-protecting agents.

Microbial metabolites continue to attract attention as potential plant protection agents because they are expected to overcome the pollution problems caused by the use of synthetic chemical pesticides (Prabavathy et al., 2006). These metabolites are

inherently biodegradable and often do not accumulate in the environment (Prabavathy et al., 2006).

Pseudomonas aeruginosa is a gram-negative bacterium. This opportunistic pathogen is capable of living in very diverse environments because of its complex genome (Stover et al., 2000). It has been shown to convert exogenously applied ricinoleic acid (RA) into 7,10,12-trihydroxy-8(E)-octadecenoic acid (TOD) (Kim, H. et al., 2002). RA is a mono-hydroxy fatty acid derived from the castor plant (Levinson et al., 2005). TOD closely resembles naturally occurring trihydroxy unsaturated fatty acids such as those produced by resistant cultivars of rice to rice blast (Kato et al., 1985, 1986). Two such naturally occurring compounds, 9S,12S,13S,-trihydroxy-10-octadecenoic acid from rice plants and 9,12,13-trihydroxy-10(E)-octadecenoic acid from *Colocasia antiguorum* are known to possess antifungal activity (Kato et al., 1985, 1986; Suemune, 1988; Masui et al., 1989). Hou (1995) found TOD to be an antifungal agent active against *Monilinia fructicola*, *Phytophthora infestans*, *Rhizoctonia solani*, and *M. grisea* the causal organisms respectively of peach blossom blight, potato late blight, rice sheath blight, and rice blast. The greatest percent of growth inhibition was reported with *M. grisea* (Hou, 1995).

Pseudomonas aeruginosa is an ideal organism to study enhanced methods of production and isolation of a natural antifungal agent because of the relative ease with which it can be grown and manipulated. Although little is know about the genes involved in the conversion process, the proposed pathway involves the hydroxylation of C-7 and C-10 which results in the rearrangement of the double bond from C 9–10 (*cis*) to 8–9

(*trans*) as illustrated in Figure 5. Further hydroxylation of the secondary dihydroxy fatty acid results in TOD (Kim, H. et al., 2002).

The objective of this research is to screen random transposon insertion mutants of *P. aeruginosa* and identify those that are potentially defective in the conversion of RA to TOD. The insertion sites of the transposons of the potential TOD^- mutants can then be sequenced to determine the genes involved in the conversion of RA to TOD. This could potentially lead to a quicker and more cost effective method of TOD production.

2. Material and Methods

2.1. Biological Materials.

Magnaporthe grisea was grown on potato dextrose agar (PDA; Fisher Scientific, PA, USA) at 30°C under a continuous dark cycle for 10 days. The culture was transferred to oatmeal agar (OMA; Fisher Scientific, PA, USA) and placed under a black light for 10 days at 25°C (Oh and Lee, 2000). Conidia were collected from the OMA cultures as needed to begin new cultures. Mycelium was increased by placing two 5mm plugs from an actively growing PDA plate culture of *M. grisea* into a 250ml flask containing 200ml of potato dextrose broth (PDB; Fisher Scientific, PA, USA). The cultures were grown on a shaker at 155 rpm for 2 weeks. Mycelium was then collected from the flasks and used in experiments.

2.2. Chemical Materials.

The 7,10,12-trihydroxy-8(E)-octadecenoic acid (TOD) was produced by Tsung-Min Kuo as previously described (Kuo et al. 1998). Briefly, RA was added to 18 h old

bacterial cultures in 30 ml of Wallen fermentation (WF) broth and shaken continuously at 200 rpm and 28˚C for 48 h. The lipids were then extracted from the acidified broth using an equal volume of methanol/ethyl acetate. A rotary evaporator was used to remove the solvent from the combined extracts. The concentrated lipid extracts were then transferred to vials and dried under a nitrogen stream. Purified TOD was dissolved in 100% ethanol for use in all of the bioassays. Ethanol and sterile distilled water controls were included with each test to account for any effect of the diluents on the organism being screened.

2.3. Assay Development and Determination of ETOH and RA Effect on M. grisea

An *in vitro* assay using 96-well microtiter plates (Cell Wells; Corning Glass Works, Corning, NY, USA) was developed to test the effects of TOD on *M. grisea* growth. Seven day old cultures of *M. grisea* were blended with PDB on high for 1 minute. The blended solution was adjusted to 10^4 fungal fragments per ml using a hemacytometer. A 190µl aliquot of the blended *M. grisea* solution was pipetted into each well of a 96-well microtiter plate. Treatments consisting of 1) 0.15µg/µl of TOD 2) 10µl of 100% EtOH and 3) untreated control were applied directly to the appropriate well using 0.2-10µl multi-channel micropipette (Fisher Scientific, PA, USA).

All treatments were randomized and replicated 3 times and the test was repeated. Microtiter plates were sealed with parafilm and placed in a 30°C incubator. Growth in the microtiter plates was visually assessed after 7 days by recording the presence (+) or absence (-) of mycelium on the surface of the PDB in each well.

Although ricinoleic acid (RA) is converted by *P. aeruginosa* into TOD it is estimated that under optimum conditions the conversion rate is only 40% (Kuo et al.,

2001). Therefore, RA may be present in bacterial supernatant so its affect on *M. grisea* was studied. To determine if RA has an inhibitory affect on *M. grisea* similar to that of TOD it was applied to the 96-well microtiter plates containing *M. grisea* prepared as mentioned earlier. Both 80% and 99% pure RA were tested. The treatments consisted of 1) 6µl/ml 80% RA, 2) 6µl/ml 99% RA, and 3) untreated control. All treatments were arranged in a randomized complete block design (RCBD) with 3 replications. Growth was visually assessed by noting the presence (+) or absence (-) of mycelium on the surface of the PDB in each well. The test was repeated.

2.4 Transposon Mutagenesis

Jessica Cofield carried out a bi-parental mating between an *Escherichia coli* donor carrying a mini Tn*5*-B21 transposon and a *P. aeruginosa* recipient (Figure 6). Mini Tn*5*-B21 was chosen because it was less likely to transpose multiple times. The transposon carried the gene encoding for tetracycline resistance (Tc^R) allowing for selection.

2.5 Inhibitory Concentration Determination

Stock assay 96-well microtiter plates of *M. grisea* were generated using the microtiter plate method mentioned previously. Various concentrations of TOD were tested in order to determine the inhibitory concentration. Concentrations from 0µg/ml to 450µg/ml were tested by adding the stock TOD solution in 90µg/ml increments for a total of 5 concentrations tested. Further testing was done with concentrations from 90µg/ml to 180µg/ml tested by adding the stock TOD solution in 15µg/ml increments for a total of 6

concentrations tested. A final concentration test was carried out with concentrations from 135µg/ml to 150µg/ml tested by adding the stock TOD solution in 3µg/ml increments for a total of 5 concentrations tested.

Untreated controls as well as ethanol controls were included in each concentration test. Both experiments were arranged in a RCBD with 3 replications, and the test was repeated. Growth was visually assessed as in the previous tests by noting the presence (+) or absence (-) of mycelium on the surface of the PDB in each well.

2.6 Isolation of Bacterial Culture Supernatants Used in Mutant Testing

TOD is present in the culture supernatant following the conversion process. Supernatants must therefore be extracted from the transposon mutants to use in the screening assay. Subcultures from the plates containing the transposon insertion mutants were made to 96-well microtiter plates containing WF broth. Plates were then incubated on a rotary shaker (Model G25 Controlled Environment Incubator Shaker, New Brunswick Scientific Co. Inc. Edison, N.J) at 30°C and 180 rpm for 24 h. RA was diluted to a 1% solution and 7.5µl was added to each well after the initial 24 h incubation period. The plates were then incubated at 30°C for an additional 24 hours on the shaker at 180 rpm. After the final 24 hour incubation period the plates were centrifuged for 30 minutes at 7,000 rpm. One hundred microliters of supernatant was removed from each well and placed into the corresponding wells of a new 96-well microtiter plate. Plates containing supernatants were either used immediately or parafilmed and frozen at -80˚C until needed.

2.7 Supernatant Concentration Determination

Pseudomonas aeruginosa strain NRRL B-800 is unable to carry out the conversion of RA into TOD while strain NRRL B-2326 is capable of carrying out the conversion. The supernatants from each of these strains were collected by the same method mentioned above and used in a concentration test in order to determine the volume of supernatant needed during the mutant screening to identify possible TOD^- mutants. The highest volume at which supernatants from NRRL B-800 (the strain unable to carry out the conversion) did not inhibit growth but those from NRRL B-23260 (the strain capable of carrying out the conversion) did would be the volume used in the mutant screening. The 96-well microtiter plate assay method was used to carry out this experiment. Concentrations of supernatants ranging from 0µl/ml - 200µl/ml per well were tested in increments of 25µl/ml. An untreated control was included and the test was arranged in a RCBD with 4 replications and repeated. Growth was visually assessed by noting the presence (+) or absence (-) of mycelium on the surface of the PDB in each well.

2.8 Mutant Identification

The 96-well microtiter plate assay was used to screen the 15,168 transposon insertion mutants created in order to identify mutants unable to carry out the conversion of RA to TOD (Figure 7). Based on the data collected in the supernatant concentration determination experiment, 26µl of supernatant from each mutant was collected and applied to a well containing 20,000 *M. grisea* fungal fragments in 174µl of PDB to achieve a final volume of 200µl. All treatments were arranged in a RCBD with 3

replications. Controls used in each round of screening, included: 1) untreated control; 2) 26µl of NRRL B-800 supernatant; and 3) 26µl of NRRL B-23260 supernatant. Due to time limitations, only 480 – 960 mutants could be screened in each round. A control plate was included with each screen to ensure that the fungal culture was still actively growing. After treatment, the plates were parafilmed and incubated for 7 days under 24 h dark cycles at 30°C. Growth was visually assessed by noting the presence (+) or absence (-) of mycelium on the surface of the PDB in each well. The wells in which growth was present in all three replications were identified as potential TOD^- mutants because the presence of growth should correspond directly to absence of TOD.

3. Results and Discussion

3.1 Assay development and determination of ethanol and RA effect on M. grisea

The 96-well microtiter plate assay required a 5-7 day incubation period at 30˚C. Both the fungus and TOD were in a liquid solution which maximized the interaction of the fungal culture with the TOD. The addition of 10µl of stock TOD solution resulted in complete growth inhibition (Table 5). The ethanol treatment did not cause any *M. grisea* growth inhibition compared to the untreated control at the concentration tested and was therefore considered a suitable solvent for TOD during the experiment. The addition of 80% pure RA inhibited the growth of *M. grisea* at the concentration tested compared to the untreated control. *Magnaporthe grisea* growth, however, was not inhibited with the addition of the same volume of 99% pure RA. This data suggested that something present in the 80% pure RA was having an inhibitory effect on the fungus. To ensure that

any growth inhibition of *M. grisea* was not caused by RA remaining in the supernatant after the conversion process only 99% pure RA was added to the transposon mutants.

3.2 Construction of a transposon library

From the transposon mutagenesis a library of 15,168 Tn*5*-B21 insertion mutants were isolated as Tc^R colonies and each Tc^R colony was frozen for future screening. The large number of mutants was generated to ensure an insertion into every gene at least once. The number was approximately three times the number of genes found in *P. aeruginosa*.

3.3 Inhibitory Concentration Determination Assay

In the initial inhibitory concentration test, complete growth inhibition of *M. grisea* was observed with the addition of 180µg/ml TOD. All concentrations tested above 180µg/ml continued to inhibit *M. grisea*. The same test was then repeated but with concentrations ranging from 90µg/ml to 180µg/ml at 15µg/ml increments. Complete growth inhibition was observed with the addition of 150µg/ml. A final test was carried out with concentrations from 135µg/ml - 150µg/ml at 3µg/ml increments. Complete growth inhibition of *M. grisea* was observed with the addition of 144µg/ml of TOD. Again all volumes tested above 144µg/ml continued to inhibit the growth of *M. grisea*. The data collected showed that for the 96-well microtiter plate assay a concentration of 144ppm of TOD completely inhibited the growth of *M. grisea* (Table 1).

3.4 Supernatant Concentration Determination

The goal of the initial mutant screen was to identify mutants unable to convert RA to TOD. Certain mutants may have been capable of producing TOD but in greatly reduced quantities. For this reason it was desirable to apply a large volume of supernatant during the mutant screening to help eliminate false identification of mutants that were not completely defective for TOD production. The supernatant concentration determination test was carried out in order to ensure that at high concentrations other compounds in the supernatant were not causing a negative effect on the growth of *M. grisea*. A concentration of 148µl/ml or more of supernatant from NRRL B-800 (the strain unable to create TOD) caused inhibition of fungal growth. The supernatant from NRRL B-23260 caused inhibition of fungal growth at a concentration of 47µl/ml and continued to cause inhibition of *M. grisea* at all higher concentrations tested (Table 2). Based on this data a concentration of 130µl/ml of supernatant was used during the mutant screen to test for the presence or absence of TOD.

3.5 Identification of Potential TOD- Mutants

The final screen revealed 107 possible TOD^- mutants of the 15,168 mutants screened (Table 3). This number was much higher than we anticipated and may be the result of multiple mutants in which the transposon inserted into the same gene. This could have been caused by the presence of hotspots in the genome. Regulators could also be disrupted causing a significant decrease in TOD production. Reduced concentrations of TOD may be unable to inhibit *M. grisea* growth.

3.6 Summary

Identification of the genes involved in the conversion of ricinoleic acid into TOD is a critical step in producing an economically competitive and environmentally friendly biological crop protection agent. Now that potential TOD$^-$ mutants have been isolated the transposon insertion points can be determined and PCR can be used to amplify genes of interest. This will allow for the identification and characterization of genes involved in TOD biosynthesis. Current research is also being conducted in order to determine the antimicrobial range of TOD.

Acknowledgments

Purified TOD was supplied by Tsung-Min Kuo with the USDA, ARS, NACUR in Peoria, Illinois. This study was supported by a grant from the Alabama Agricultural Experimental Station to Suh and Lawrence (AAES-FG-06-G).

FIGURES

Figure 5. Schematic of proposed conversion pathway by which *Pseudomonas aeruginosa* PR3 in converts ricinoleic acid into 7,10,12-trihydroxy-8(E)-octadecenoic acid.

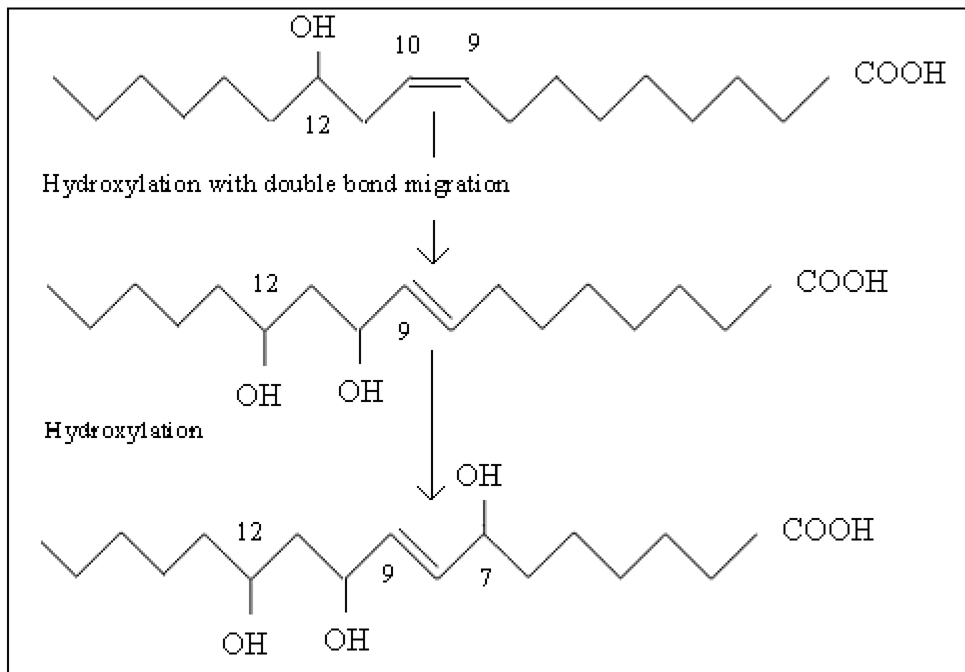


Figure 6. Bi-parental mating between *Escherichia coli* donor and *Pseudomonas aeruginosa* recipient.

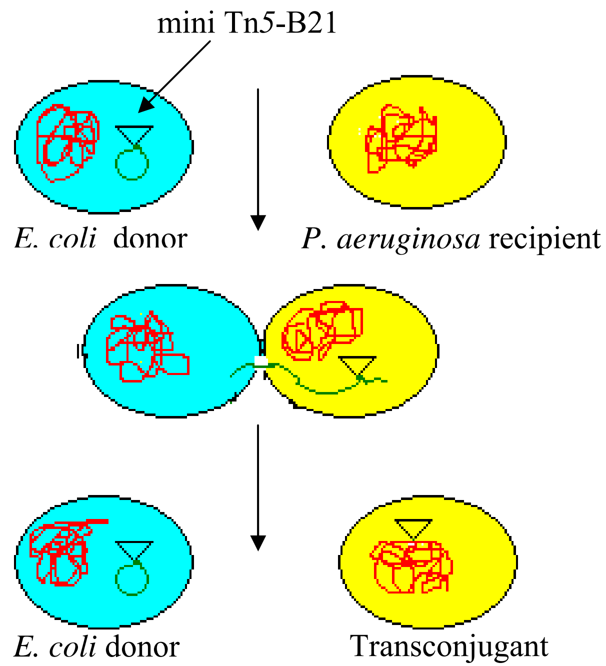
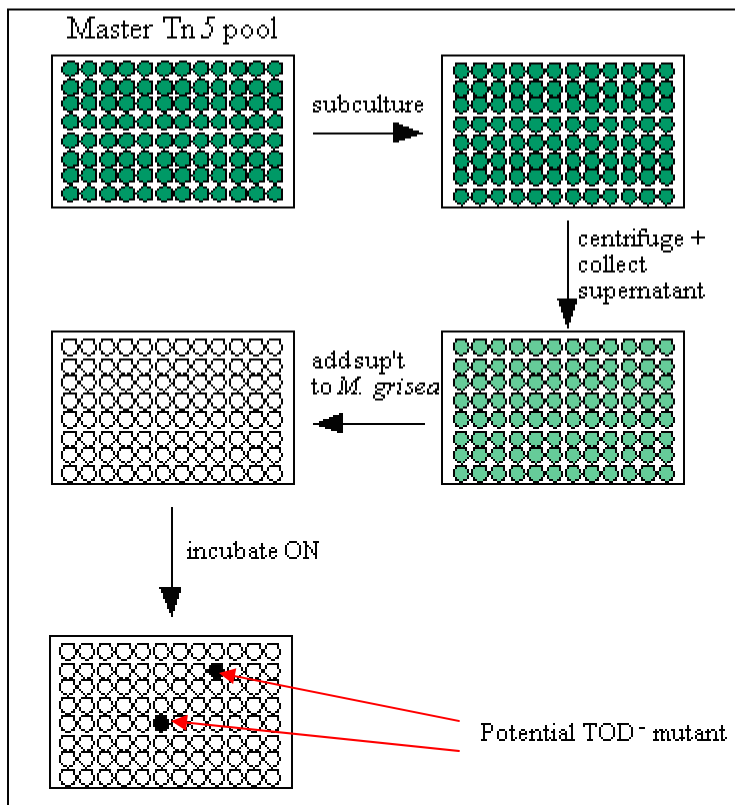


Figure 7. Diagram of genetic screen for TOD$^-$ mutants.



TABLES

Table 1. Inhibitory concentration of TOD (µg /ml) against *Magnaporthe grisea* using the microtiter plate assay.

	TOD Concentration (µg /ml)					
	142.8	145.0	148.3	150.0	153.6	156.0
M. grisea	1	1	1	0	0	0

0 = no mycelial growth
1 = mycelial growth

Table 2. *Magnaporthe grisea* mycelial growth as affected by supernatants from *Pseudomonas aeruginosa* strains NRRL B-800 (800) and NRRL B-23260 (23260) at increasing volumes.

	Volume of Supernatant Added (μl)										
Strain	0	24	47	69	90	111	130	148	166	183	200
800	1	1	1	1	1	1	1	0	0	0	0
23260	1	1	0	0	0	0	0	0	0	0	0

0 = no mycelial growth
1 = mycelial growth

Table 3. Plate designation of potential TOD$^-$ mutants identified.

Plate 2	**Plate 3**	**Plate 4**	**Plate 5**	**Plate 6**
A: 9, 11	A: 3, 7, 8, 12	C: 3, 10	A: 1, 5, 12	A: 10
B: 3, 4, 5, 8, 9, 10, 11	B:5, 6, 10	D: 11	B: 5, 11, 12	B: 7, 11
C: 2, 3, 4, 5, 6, 10	C: 5	F: 2	C: 1	C:2
D: 5, 8	E: 1, 2	G: 3, 6	D: 12	D: 1
E: 2, 5, 7, 12	F: 1, 2, 3	H: 2, 8	E: 1	E: 6, 9
G: 6	G:1, 2		F: 12	G: 8
H: 12	H: 12		G: 10	H: 1, 6, 7
			H: 11	
Plate 7	**Plate 8**	**Plate 9**	**Plate 11**	**Plate 12**
A: 3, 4, 11	G: 6, 8	A: 2, 8	F: 8	B: 7
B: 7			H: 12	
E: 3				
G: 5, 7, 8, 11				
H: 3, 10				
Plate 14	**Plate 17**	**Plate 19**		
A:4	**F**: 10	**E**: 10, 11, 12		
B: 5		**F**: 11		
C: 8		**H**: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12		
E: 10				

II. SURVEY OF THE BIOLOGICAL ACTIVITY OF A NOVEL ANTIFUNGAL COMPOUND PRODUCED BY *PSEUDOMONAS AERUGINOSA*

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Abstract

The trihydroxy fatty acid 7,10,12-trihydroxy-8-octadecenoic acid (TOD), a known plant self-defense substance (Masui et al., 1989), was tested for antimicrobial activity against 23 species of fungi, 17 species of bacteria, and 2 species of nematodes. The antifungal activity of TOD was determined by the visual presence or absence of mycelial growth. Antibacterial activity was determined by lawn streaking tryptic soy agar (TSA) petri plates with an individual bacterial species, spot applying TOD to the plate and measuring the zone of inhibition. Potential effect of TOD on nematode mobility was determined in 12-well microtiter plates. Cotton root colonization by the two species of nematodes was also tested after exposure to TOD.

Magnaporthe grisea growth was completely inhibited by TOD at 150µg/ml. At this concentration, mycelial growth of the remaining fungal species screened was not

completely inhibited. Even with the addition of 0.025 ppm of TOD, total growth inhibition in vitro was not observed in *Rhizoctonia solani* or *Phytophthora infestans*. Of seventeen bacterial species screened, growth of fifteen species was significantly inhibited with addition of 150µg/ml of TOD compared to the ethanol control. The average zone of inhibition ranged from 1.50cm – 2.01cm. *Meloidogyne incognita* and *Rotylenchulus reniformis* nematode mobility, as well as cotton root colonization, was unaffected by TOD.

Our data suggest that TOD has potential to be used as a selective biocontrol agent against the fungal pathogen, *M. grisea,* and as a non-selective biocontrol agent against various bacterial plant pathogens.

Introduction

Plant defense mechanisms are often used as model systems when developing new methods of disease management and pest control. Many natural products are used commercially as crop protection agents (Copping and Duke, 2007). Rice, taro, and tomato are examples of plants that have been shown to create antifungal oxylipins, which are oxygenated fatty acids, as part of their defense system (Kato et al., 1983; Masui et al., 1989 and Vernenghi et al., 1986). Kato et al. (1983) found that rice plants resistant to rice blast produce antifungal substances. The antifungal substances isolated consisted of several kinds of oxygenated unsaturated fatty acids (Kato et at., 1984). The amount of these enzymes produced by the plant depended largely on a combination of rice cultivar and race of the fungus (Kato et al., 1986). Masui et al. (1989) isolated antifungal compounds from taro tubers inoculated with the sweet potato strain of *Ceratocystis*

fimbriata, the causal agent of the fungal disease black rot of sweet potato. Fifty parts per million (ppm) of 9,12,13-trihydroxy-(E)-10-octadecenoic acid isolated from inoculated taro tubers completely inhibited the germination of conidia of *C. fimbriata*. Dellar et al., (1996) isolated 4,9-diacetoxy-6,7-epoxy-5-hydroxy-8-oxododeca-2-enoic acid and 4,6,7,9-tetraacetoxy-5-hydroxy-8-oxododeca-2-enoic acid, two polyoxygenated fatty acids with antifungal activity from the aerial parts of the shrub *Aeollanthus parvifolium* (Labiatae). Both compounds inhibited conidial germination of the fungus *Cladosporium cucumerinum* which causes cucurbit scab.

Microorganisms are also capable of producing hydroxy fatty acids by the biotransformation of unsaturated fatty acids (Hou, 1995). They are classified as one of the following: monohydroxy (Hou, 1994; Hou and Bagby, 1992); dihydroxy (Hou and Bagby, 1991); and trihydroxy fatty acids (Hou, 1996). Many of these have demonstrated antimicrobial properties. Benyagoub et al., (1996) characterized two new fatty acids with antifungal activity from *Sporothrix flocculosa*, a biocontrol fungus shown to inhibit powdery mildew on both roses and cucumbers (Bélanger et al., 1994; Hajlaoui and Bélanger, 1991; Jarvis et al., 1989). Hou and Forman (2000) tested three types of hydroxy fatty acids for anti-plant pathogenic fungal activity. A 200 ppm concentration test compound solution was sprayed on plants and after 24 h the plants were inoculated with the following pathogenic fungi: *Botrytis cinerea, Erysiphe graminis*, *Phytophthora infestans*, *Pseudocerocosporella herpotrichoides*, *Puccinia recondite*, *Pyricularia grisea, Rhizoctonia solani,* or *Septoria nodorum*. Both 10-hydroxystearic acid (HAS) and 7S,10S-dihydroxy-8(E)-octadecenoic acid (DOD) showed no disease control activity. However, 12,13,17-trihydroxy-9(Z)-octadecenoic acid (THOA) suppressed, although

weakly, the disease expressed by: *E. graminis*; *P. recondite*; *P. infestans*; and *B. cinerea* by 77, 86, 56, and 63%, respectively, compared to untreated controls. Hou and Forman (2000) concluded that the specificity of trihydroxy fatty acids against certain plant pathogenic fungi may depend on the location of the hydroxy groups on the trihydroxy fatty acid molecule.

Pseudozyma flocculosa is a fungus that inhibits the growth of powdery mildew fungi (Jarvis et al., 1989). The fatty acid cis-9-heptadecenoic acid (CHDA) produced by *P. flocculosa* was found to be responsible for the antifungal properties (Benyagoub et al., 1996). Avis and Belanger (2000) studied the biological characteristics of CHDA, and although degree of sensitivity varied considerably, the greatest in vitro growth inhibition was seen with *P. infestans* (64.9%) and *Pythium aphanidermatum* (66.8%).

Walters et al. (2004) examined in vitro effects of the fatty acids linolenic acid, linoleic acid, erucic acid and oleic acid on growth of the plant pathogenic fungi *R. solani*, *Pythium ultimum*, *Pyrenophora avenae* and *Crinipellis perniciosa*. In that study, linolenic acid reduced the mycelial growth of *R. solani* and *C. perniciosa* at 100µM but the concentration had to be increased to 1000µM before there was any effect on the mycelial growth of *P. ultimum* and *P. avenae.* The second acid studied, linoleic acid, significantly reduced the mycelial growth of *C. perniciosa* at 100µM and *R. solani*, *P. ultimum*, and *P. avenae* at 1000µM. Oleic acid was found to only significantly reduce mycelial growth of *P. ultimum* at 100µM and *C. perniciosa* at 1000µM. The last acid, erucic acid did not reduce the mycelial growth of any of the fungi tested at either concentration (Walters et al., 2004).

Kuo et al. (1998) investigated the bioconversion of ricinoleic acid by *Pseudomonas aeruginosa* PR3 and determined the new compound produced by the transformation was 7,10,12-trihydroxy8(E)-octadecenoic acid (TOD). Kuo et al. (2001) investigated the biological activity of TOD which resembles hydroxy fatty acids produced by rice plants as self defense substances (Kato et al., 1984). *Pseudomonas aeruginosa* PR3 NRRL B-18602 was utilized for the bioconversion. To test the antifungal activity of TOD against plant pathogenic fungi, a concentration of 5 ppm of TOD was formulated and sprayed onto each test plant which was inoculated with the pathogenic fungus 24 h later. Test plants were incubated 34 - 48 h and rated 4 to 5 days after inoculation. TOD was active against *Monilinia fructicola*, *P. infestans*, *R. solani*, and *M. grisea*, the causal organisms respectively of peach blossom blight, potato late blight, rice sheath blight and blast. TOD did not show any activity against the causal organisms of wheat foot rot (*Fusarium* spp.) and wheat glume blotch (*Septoria* spp.). Among the positive tests, TOD had the best activity (29% disease suppression) against *M. grisea*.

Kuo et al. (2001) also investigated the production of TOD by *P. aeruginosa*. TOD was produced by *P. aeruginosa* PR3 (NRRL B-1862) in a culture supplied with exogenous ricinoleic acid. The yield of TOD production was always higher in a rich culture medium than in minimal media. Extending the conversion time from 48 to 72 h prior to lipid extraction led to a 65% reduction in yield, indicating that TOD was further metabolized by strain PR3 and that control of conversion time was important to achieving a maximum yield. The optimum culture density, reaction time, pH, temperature, and substrate concentration for production of TOD were: 20 - 24 h culture growth, 48 h, pH

7.0, 25°C, and 1% (vol/vol), respectively. Under optimum conditions, TOD production yield was greater than 45%. Kuo and Nakamura (2004) studied 16 *P. aeruginosa* strains for their fatty acid converting abilities. All strains exhibited varying levels of TOD production, with strains NRRL B-1000 and NRRL B-23260 producing the most. They concluded that production of significant quantities of TOD from ricinoleic acid appeared to be a characteristic trait of *P. aeruginosa*.

The purpose of this experiment is to learn more about the antimicrobial activity of TOD. Various species of plant pathogenic bacteria, fungi, and nematodes were screened to test for sensitivity toward TOD to provide a better understanding of the characteristics of TOD and its potential use as a biocontrol agent.

2. Materials and Methods

2.1. Biological materials

A range of fungi, bacteria, and nematodes were included to test for potential species specific effects of TOD. The fungal species screened were indigenous fungal isolates from the southern U.S. region collected from diseased cotton, soybean and corn field plants in Alabama. Cultures were maintained on potato dextrose agar (PDA; Fisher Scientific, PA, USA) slants or in skim milk (Fisher Scientific, PA, USA) microtubes stored at 4.4°C and -6.7°C. Bacterial species tested in the bioassays were from Dr. Joseph Kloepper's Lab culture collection at Auburn University and were a representation of plant pathogenic as well as beneficial bacteria commonly found in the same region. The bacterial cultures were frozen at -80ºC and were subcultured as needed. Subcultures were made by transferring a portion of the frozen bacterial culture to flasks containing

150 ml of tryptic soy broth (TSB; Fisher Scientific, PA, USA). Flasks were maintained on a shaker at 155rpm and 30°C. *Rotylenchulus reniformis* and *Meloidogyne incognita* were collected from fields across the state of Alabama and represent two nematode species which cause devastating crop loss in the southeastern U.S. Nematodes were maintained in the greenhouse on cotton cultivar Delta and Pine Land 555 BG/RR grown in a 500 cc polystyrene pots containing sterilized loamy sand (72.5%, 15%, 12.5%, S-S-C, pH 6.4) at day and night temperatures ranging from 26.7°C to 22.2°C respectively.

2.2. Chemical materials.

The 7,10,12-trihydroxy-8(E)-octadecenoic acid (TOD) was purified as previously described (Kuo et al. 1998). Purified TOD was dissolved in 100% ethanol for use in all bioassays. Ethanol and sterile distilled water controls were included with each test to determine effects of diluents on the organism being screened.

2.3. In vitro antifungal screens

Antifungal activity of TOD was tested on 23 fungal species using a 96-well microtiter plate (Cell Wells; Corning Glass Works, Corning, NY, USA) assay modified from Capilla et al. (2001). The selected fungi (Table 4) were grown independently on appropriate medium to increase hyphal growth. An agar plug (3 mm diameter) was cut from the actively growing edge of a 10 day old fungal culture and placed into the wells of a 96-well microtiter plate. In order to hydrate the fungal plug, 150µl of potato dextrose broth (PDB; Becton and Dickinson Co., MA, USA) was added to each well. All fungal species were exposed to the following treatments: 1) 0.1875µg/µl TOD; 2) 10µl of 100%

EtOH; or 3) 10µl of sterile distilled water. The antifungal assays were statistically arranged in a randomized complete block design (RCBD) with 3 replications. Plates were incubated for 5 days at 30°C, and a mycelium growth rating of + (presence) or – (absence) assigned. The initial screen was conducted with one concentration of TOD and was intended to screen for fungi with similar sensitivity as *M. grisea* to TOD. The entire fungal screen was repeated three times. An additional screen was carried out in the same manner mentioned above but with an increased concentration of TOD on *R. solani* and *P. infestans*. This additional screen was conducted because previous research (Kuo et al., 2001) showed these two fungi to be sensitive to TOD. The concentration of TOD was increased from 0.1875µg/µl to 1.875µg/µl. Ethanol and sterile distilled water controls were also included.

2.4. In vitro antibacterial screens

Antibacterial activity of TOD was tested on 17 different species of bacteria (Table 5). The species screened included gram positive (+) and gram negative (-) bacteria that were categorized as either plant pathogenic or plant growth promoting. The bacterial cultures were incubated on a rotary shaker (Model G25 Controlled Environment Incubator Shaker, New Brunswick Scientific Co. Inc. Edison, N.J.) in test tubes containing 1mL of tryptic soy broth (TSB; Becton and Dickinson Co., MA, USA) at 30°C and 180 rpm for 24 h. After the incubation period, 20µl of the bacterial culture broth was diluted with 180µl of 0.7% saline solution in order to lower the concentration of bacterial cells. Twenty microliters of the diluted bacterial solution was spread on triplicate plates containing 20ml of TSA and allowed to dry for 15 min. All three

treatments were spot applied on each of the plates, equal distances from each other. Treatments consisted of: 1) 30µg of TOD; 2) 10µl of 100% EtOH; and 3) 10µl sterile distilled water. Plates were arranged in a RCBD, replicated three times and incubated at 30°C for 24 h. Zones of bacterial growth inhibition were measured at right angles and recorded (Figure 8). The entire test was repeated four times.

2.5. Nematicidal screens

Meloidogyne incognita and *R. reniformis* were increased in the greenhouse on cotton for 45-60 days. *Rotylenchulus reniformis* juveniles and vermiform adults and *M. incognita* J2's were extracted from the soil by a combination of gravity sieving and sucrose centrifugation and enumerated with a dissecting microscope (Jenkins, 1964; Diez et al., 2003). The final volume was adjusted to 6000 vermiform life stages and eggs per ml of water. Toxicity screens were conducted in 12-well microtiter plates with each well receiving 600 µl of the nematode solution containing 3600 vermiform life stages and eggs. Thirty nematodes in each well were tactically stimulated using a root canal probe to determine viability before testing compounds. Treatments included: 1) sterile water control; 2) 60 ppm aldicarb (Temik® Bayer CropScience, Research Triangle Park, North Carolina); 3) 30 ppm oxamyl (Vydate® DuPont, Wilmington, Delaware); 4) 2.48µg/µl of TOD; or 5) 5µl of 100% EtOH. Plates were arranged in a RCBD and replicated four times. Two hours after inoculation, nematodes were again tactically stimulated in order to determine mobility. Nematodes that did not move were considered dead.

In order to determine the effect of TOD on nematode infectivity, the total solution in each well was increased to 1ml and pipetted into two 1-cm depressions on either side

of a 5 day old cotton seedling. Seedlings were grown in the greenhouse in a loamy sand (72.5%, 15%, 12.5%, S-S-C, pH 6.4). Nematode infectivity and developmental stage were determined seven days after inoculation by collecting and staining the roots using acid fuchsin (Byrd et al., 1983). The stained nematodes were enumerated using a dissecting microscope. The life stages of the nematodes present were determined and recorded based on Diez et al. (2003). The entire in vitro and greenhouse tests were carried out three times.

2.6. Statistical Analysis

Fungal data recorded by visually noting the presence or absence of growth were not analyzed statistically since there was no variation among replications and all tests produced similar results. Zone of inhibition data from the in vitro antibacterial screens was analyzed as a complete factorial of bacterial strain (17) and treatment (2 – TOD in EtOH vs. pure EtOH) within an RCBD (r = 3). The entire experiment was conducted twice. Mixed Models methodology as implemented in SAS® Proc GLIMMIX (http://support.sas.com/rnd/app/da/glimmix.html) was employed. Experimental repeat and blocks were treated as random effects, whereas bacterial strain, treatment, and their interaction were considered to be fixed as fixed. Residuals followed a Gaussian distribution. We used the slicediff option to calculate the P-values for simple effect of treatment with bacterial strain. The simulation option was used in the comparison of bacterial species within treatment to guard against an inflated Type I error rate.

Nematode data were analyzed using generalized linear models procedures as implemented in SAS Proc GLIMMIX (http://support.sas.com/rnd/app/da/glimmix.html).

Experimental repeats and replicates were treated as random effects. Nematode counts per gram of root where well behaved and could be modeled with the normal distribution function, but analysis of treatment effect on developmental stage required lognormal distribution. Nematode survival data were modeled using the binary distribution function. Heterogeneous variance among treatments was addressed by R-side modeling using the group option within the random statement. Nematicide treatments were absolute effective with 100% mortality and zero variance. By definition then, all other treatments whose confidence intervals did not include 100 were considered to be different from these two treatments. A similar argument can be made for the untreated control which had a perfect zero mortality. The statistical analysis thus reduces to a simple contrast between treatments TOD and EtOH. Similarly, data from the nematode colonization experiment do not contain nematicide treatments, as all nematodes in those treatments were killed. Contrasts were used to evaluate the differences among treatments.

3. Results and Discussion

3.1. TOD's antifungal activity

Magnaporthe grisea growth was completely inhibited by TOD at the concentration of 0.01 ppm (Table 6). The three tests produced similar results with no interactions over time. Total growth inhibition was not observed in any of the remaining fungal species screened. Growth of two of the fungi screened, *R. solani* and *P. infestans*, had been shown by Kuo et al. (2001) to be inhibited 21% and 1% respectively when test plants were sprayed with 5 ppm of TOD in a growth chamber evaluation. Data collected

from the microtiter plate assay in the present study showed that with the addition of 1.875µg/µl of TOD total growth inhibition in vitro did not occur in *R. solani* and *P. infestans*. Data collected in this experiment revealed that although *R. solani* and *P. infestans* have demonstrated sensitivity toward TOD in previous studies, they are not as sensitive as *M. grisea* and their growth is not completely inhibited by TOD.

Previous research suggests that location of the hydroxyl group on the fatty acid makes it very specific in its effect on the target organism (Hou and Forman, 2000). Hou and Forman (2000) screened three different hydroxy fatty acids and found only one capable of inhibiting a small range of fungi and total growth inhibition was not observed.

Although the exact mechanism of fungicidal activity of trihydroxy fatty acids remains undetermined it is known that fatty acids can inhibit cellular respiration in the mitochondria (Lewis and Johnson, 1968). Nam et al. (2004) hypothesized that fatty acids are incorporated into the hydrophobic domain of the membrane because they are partially soluble in the membrane phospholipids. There may be considerable variation even among closely related strains due to partial incorporation of fatty acids through membrane lipid biosynthesis (Garg and Müller, 1993). When studying CHDA, Avis and Belanger (2000) concluded that fungi sensitivity varied as a result of specific partitioning into the fungal membrane, and was also strongly affected by the sterol content of a particular fungus. Sensitive fungi exhibited higher membrane disorder which resulted in conformational alterations in membrane proteins and ultimately resulted in higher membrane permeability (Avis and Belanger, 2000). Sensitivity variation toward TOD demonstrated in the fungi tested may be due to differing sterol contents of the fungi and varying degrees of membrane incorporation of TOD.

The assay used in this experiment was designed to assess the antifungal activity of TOD using the conditions that were effective against *M. grisea* in vitro. Further screening using higher concentrations of TOD may need to be conducted to determine if TOD is active against other fungi at increased concentrations.

3.2. TOD's Antibacterial Range.

Fifteen of the seventeen bacterial species screened exhibited significantly greater inhibition with TOD than with the ethanol control ($P \leq 0.05$) (Table 7). The bacterial species showed varying degrees of sensitivity toward TOD with zones of inhibition ranging from 1.50cm – 2.01cm (Figure 8). In the bioassays TOD inhibited both plant pathogenic and plant growth promoting bacteria. The data collected showed there was no species specificity exhibited by TOD and both gram + and gram – species were inhibited.

There was no difference in sensitivity to TOD among 10 of the bacteria tested. *Bacillus pumilu* AP-03, *Clavibacter michiganensis*, *Pectobacterium carotovorum subsp atrosepticum*, *Pectobacterium carotovorum subsp carotovorum*, *P. putida* AP-01, *P. putida* AP-19, *P. syringae pv. lachrymans*, *Rhizobium radiobacter* 89C-02, and *Xanthomonas campestris pv campestris* were all similarly sensitive to TOD ($P \leq 0.0001$) with an average zone of clearing of 1.89 cm. *P. syringae pv tabaci* ($P = 0.0014$), *R. radiobacter* 89C-01 ($P = 0.0109$), *R. rhizogenes* ($P = 0.0014$), *X. axonopodis pv malvacearum* ($P = 0.0215$), and *X. vesicatoria* ($P = 0.0019$) were all significantly inhibited compared to the ethanol control but were less sensitive than the 10 bacterial species mentioned previously. Growth of both *B. pumilus* AP-23 and *P. syringae pv tomato* was not significantly inhibited by TOD compared to the ethanol control ($P \geq$

0.05). Interestingly, the two strains of *B. pumilus* and *P. syringae* exhibited significant differences in sensitivity. Varying sensitivity to fatty acids among various strains of *Pseudomonas* was demonstrated by Prost et al. (2005) who screened 7 different bacterial species for sensitivity to plant oxylipins. Of the 7 bacterial species screened, 4 were species of *Pseudomonas*. Varying degrees of sensitivity among the 4 species of *Pseudomonas* were observed with 21 out of the 47 oxylipins tested. Ouattara et al. (1997) tested the antibacterial activity of 8 fatty acids against 6 different bacterial species. In their experiment, lauric, palmitoleic, linoleic, and linolenic acids exhibited various inhibitory activity against bacteria with lauric and palmitoleic acids having the greatest effect (Ouattara et al., 1997).

Research has shown certain fatty acids to be capable of inhibiting gram negative (-) bacteria in addition to gram positive (+) (Karapinar and Aktug, 1987; Shin et al., 2007). Shin et al. (2007) used an agar disc diffusion assay to evaluate the antimicrobial activity of a long-chain polyunsaturated fatty acid on several food borne and food spoilage pathogens. Growth of *B. subtilus*, *Listeria monocytogenes*, *Staphylococcus aureus* and *P. aeruginosa* was inhibited by the fatty acid. The minimum inhibitory concentration varied between 500-1350 g/ml with the different species screened (Shin et al., 2007).

The exact mode of action for TOD is currently not known. In the bacterial screening experiments, TOD zones' of clearing only varied between 1.5 cm and 2.02 cm. Ethanol, however, was much more variable with zones of clearing ranging from 0.45 cm to 1.55 cm. These results suggest that TOD inhibits growth by affecting a function that is shared by all of the bacteria isolates screened. Bergsson (1999) hypothesized that lipids

kill bacteria by disruption of their cell membranes and for this reason believes that the emergence of resistant strains would be highly unlikely. Mundt et al. (2003) concluded that unsaturated hydroxylated fatty acids are capable of changing the permeability of the cell membrane, interacting with proteins and lipids of the cell membrane, and inhibiting special enzymes. Ability of a lipid to change permeability of the cell membrane would make it potentially effective across a wide range of bacterial species. Again, it is important to note that much of the difference was not in growth of bacteria treated with TOD but with ethanol.

3.3. Nematicidal screen

Rotylenchulus reniformis and *M. incognita* mortality was unaffected by TOD after 2 h exposure as compared to the untreated control ($P > 0.69$ and $P > 0.38$, respectively) (Table 8). Mortality of both nematodes species reached 100% after exposure to aldicarb and oxamyl. No variation of mortality was observed between males, females or juveniles of *R. reniformis* when exposed to TOD, aldicarb, or oxamyl.

TOD did not significantly affect the total number of *R. reniformis* ($P \geq 0.14$) or *M. incognita* ($P \geq 0.95$) per gram of root compared to the untreated control (Table 9). Neither was there any statistically significant difference between TOD and its carrier EtOH in both the *R. reniformis* ($P \geq 0.37$) and *M. incognita* ($P \geq 0.78$) tests. No *R. reniformis* or *M. incognita* infective stages were observed colonizing the cotton root tissue from the aldicarb or oxamyl treatments. The *R. reniformis* J2's (Stage A) exposed to the TOD, ethanol, and untreated treatments all developed into the Stage B females with the swollen vulva region (Table 10). Fewer Stage B females were present after treatment

with both TOD and ethanol compared to the untreated control. Although *M. incognita* J2's (Stage A) were able to enter the root, none of them developed into the Stage B sausage-shaped juveniles. Root weights were greater with the aldicarb treatment as compared to the untreated control for *R. reniformis* which is likely due to the absence of nematodes (Figure 9). There was no root colonization by nematodes treated with aldicarb or oxamyl.

Nematodes were included in the screen because Stadler et al. (1994) demonstrated that the fatty acid coriolic acid has nematicidal activity against the saprophyte *Caenorhabditis elegans*. Faske and Starr (2006) conducted experiments to determine the LD_{50} of the biological nematicide abamectin. They found that *M. incognita* was more sensitive to abamectin than *R. reniformis* likely due to the thick protective cuticle that covers *R. reniformis*. TOD may not have had an affect on the reniform nematode because of the protective cuticle; however, it also showed no nematicidal activity against *M. incognita* which does not have the same thick protective cuticle layer.

3.4. Summary

TOD is an effective antifungal agent against *M. grisea* and also showed strong antibacterial activity against 15 different bacterial species. TOD appears to have no nematicidal activity against *R. reniformis* and *M. incognita*. The use of TOD as a biological control agent of rice blast and many bacterial diseases seems promising. Current research is underway to determine minimum inhibitory concentrations for the bacteria screened, as well as identify other sensitive species. Research is also being

conducted to identify the genes involved in the conversion of ricinoleic acid to TOD by *P. aeruginosa*.

FIGURES

Figure 8. *Rhizobium radiobacter* 89C-02 from bacterial screen showing zone of inhibition by TOD.

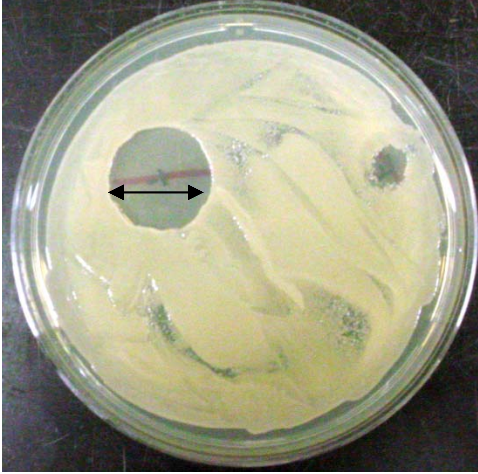
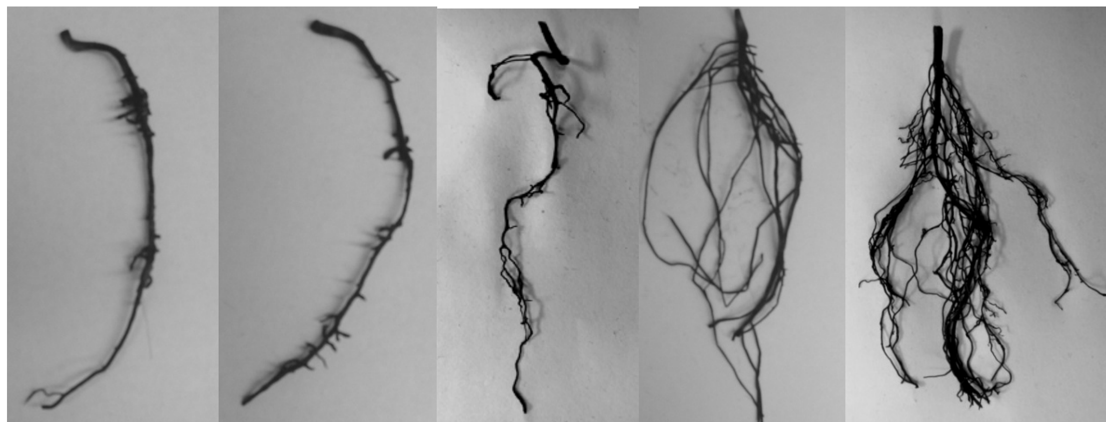


Figure 9. Fourteen day old cotton roots inoculated with *Meloidogyne incognita* and treated with TOD, ethanol, oxamyl, and aldicarb.



Untreated Ck **TOD** **Ethanol** **Oxamyl** **Aldicarb**

TABLES

Table 4. Fungal isolates screened, classification and common disease they cause.

Fungi	Class	Plant disease
Alternaria alternata	Deuteromycete/Ascomycete	Leaf spots, blights
Aspergillus niger	Deuteromycete/Ascomycete	Black mold
Aspergillus flavus	Deuteromycete/Ascomycete	Ear rot of corn
Botryosphaeria theobromae	Ascomycete	Cotton boll rot
Chaetomium aureum	Ascomycete	Soil inhabitant
Colletotrichum citrus	Deuteromycete/Ascomycete	Anthracnose
Colletotrichum dematium	Deuteromycete/Ascomycete	Anthracnose
Curvularia lunata	Deuteromycete	Leaf spot
Epicoccum purpurascens	Deuteromycete	Soil inhabitant
Fusarium moniliforme	Deuteromycete/Ascomycete	Seedling disease
Fusarium oxysporum sp. *vasinfectum*	Deuteromycete/Ascomycete	Fusarium wilt
Gliocladium catenulatum	Deuteromycete	Beneficial
Magnaporthe grisea	Deuteromycete/Ascomycete	Rice blast
Penicillium variable	Deuteromycete/Ascomycete	Soft rot on citrus
Phomopsis gossypii	Deuteromycete	Cotton boll rot
Phytophthora infestans	Oomycete	Late blight
Pythium aphanidermatum	Oomycete	Seedling disease
Rhizoctonia solani	Basidiomycete	Crown rot/seedling disease
Rhizopus stolonifer	Zygomycete	Black mold
Sclerotium rolfsii	Basidiomycete	White mold
Sordaria fimicola	Ascomycete	Soil inhabitant
Thielavia terricola	Ascomycete	Cotton boll rot
Trichoderma harzianum	Deuteromycete	Soil inhabitant

Table 5. Bacteria genus species and classification codes of isolates screened.

Isolate	Classification
Bacillus pumilus AP-03	Biocontrol agent
Bacillus pumilus AP-23	PGPR[1] strain
Clavibacter michiganensis	Plant pathogen
Pectobacterium carotovorum subsp *atrosepticum*	Plant pathogen
Pectobacterium carotovorum subsp *carotovorum* 89B-114	Plant pathogen
Pectobacterium carotovorum subsp *carotovorum* 89B-115	Plant Pathogen
Pseudomonas putida AP-01	Biocontrol agent
Pseudomonas putida AP-19	PGPR strain
Pseudomonas syringae pv *tabaci*	Plant pathogen
Pseudomonas syringae pv *tomato*	Plant pathogen
Pseudomonas syringae pv *lachrymans*	Plant pathogen
Rhizobium radiobacter 89C-01	Plant pathogen
Rhizobium radiobacter 89C-02	Plant pathogen
Rhizobium rhizogenes	Plant pathogen
Xanthomonas campestris pv *campestris*	Plant pathogen
Xanthomonas campestris pv *malvacearum*	Plant pathogen
Xanthomonas vesicatoria	Plant pathogen

[1]= plant growth promoting rhizobacteria

Table 6. Effect of TOD on fungal isolates as measured by mycelium growth.

Fungi	Inhibition
Alternaria alternata	>0.1875
Aspergillus niger	>0.1875
Aspergillus flavus	>0.1875
Botryosphaeria theobromae	>0.1875
Chaetomium aureum	>0.1875
Colletotrichum citrus	>0.1875
Colletotrichum dematium	>0.1875
Curvularia lunata	>0.1875
Epicoccum purpurascens	>0.1875
Fusarium moniliforme	>0.1875
Fusarium oxysporum	>0.1875
Gliocladium catenulatum	>0.1875
Magnaporthe grisea	**<0.1875**
Penicillium variable	>0.1875
Phomopsis gossypii	>0.1875
Phytophthora infestans	>1.8750
Pythium aphanidermatum	>0.1875
Rhizoctonia solani	>1.8750
Rhizopus stolonifer	>0.1875
Sclerotium rolfsii	>0.1875
Sordaria fimicola	>0.1875
Thielavia terricola	>0.1875
Trichoderma harzianum	>0.1875

* >0.1875 indicates that inhibition did not occur at the concentration of 0.1875 µg/µl.
* >1.875 indicates that inhibition did not occur at the concentration of 1.875 µg/µl.

Table 7. Effect of TOD and ethanol (EtOH) on bacterial isolates' growth as measured by the zone of clearing.

Bacteria	Gram sign	Average Zone of Clearing (cm)		Comparison TOD vs. EtOH
		TOD	EtOH	(P > t)[a]
Bacillus pumilus AP-03	+	2.0167	0.6167	<0.0001
Bacillus pumilus AP-23	+	1.8333	1.5000	0.0864
Clavibacter michiganensis	+	1.9167	1.0667	<0.0001
Pectobacterium carotovorum subsp *atrosepticum*	-	1.8167	0.5000	<0.0001
Pectobacterium carotovorum subsp *carotovorum* 89B-114	-	2.0000	1.1333	<0.0001
Pectobacterium carotovorum subsp *carotovorum* 89B-115	-	1.9167	0.7167	<0.0001
Pseudomonas putida AP-01	-	1.8500	0.8167	<0.0001
Pseudomonas putida AP-19	-	1.7333	0.4500	<0.0001
Pseudomonas syringae pv *tabaci*	-	1.7833	1.1500	0.0014
Pseudomonas syringae pv *tomato*	-	1.8500	1.5500	0.1222
Pseudomonas syringae pv *lachrymans*	-	1.6000	0.1000	<0.0001
Rhizobium radiobacter 89C-01	-	1.9167	1.4167	0.0109
Rhizobium radiobacter 89C-02	-	2.0167	0.9167	<0.0001
Rhizobium rhizogenes	-	1.9333	1.3000	0.0014
Xanthomonas campestris pv *campestris*	-	1.9833	0.8500	<0.0001
Xanthomonas axonopodis pv *malvacearum*	-	1.5000	1.0500	0.0215
Xanthomonas vesicatoria	-	1.9167	1.3000	0.0019

[a] Contrast of *P*-values

Table 8. Nematicidal activity of TOD on *Rotylenchulus reniformis* (reniform) and *Meloidogyne incognita* (root-knot) after 2 h of exposure in vitro. Means and standard errors (SE) are given as log(counts).

	Immobile[a]	SE [b]	Comparison
Rotylenchulus reniformis			TOD vs. Ethanol (P > t)[c]
Untreated	0	0	
TOD	3.85	± 0.39	0.69
Ethanol	4.08	± 0.42	
Aldicarb	30.0	0	
Oxamyl	30.0	0	
Meloidogyne incognita			
Untreated	0	0	
TOD	4.19	± 0.41	0.38
Ethanol	3.78	± 0.36	
Aldicarb	30.0	0	
Oxamyl	30.0	0	

[a]Mean number of nematodes out of 30 that did not respond to root canal probe stimulation.
[b] SE = standard error
[c] Contrast of *P*-values

Table 9. Effect of TOD on ability of *Rotylenchulus reniformis* and *Meloidogyne incognita* to colonize cotton roots.

	Total/gram[a]	SE[b]	Comparison (P > t)[c]		
			U[d] vs. T[e]	U vs. E[f]	T vs. E
Rotylenchulus reniformis					
Untreated	76.11	± 8.39	0.14	0.62	0.37
TOD	69.69	± 9.74			
Ethanol	57.98	± 8.70			
Aldicarb	0.0	0.0			
Oxamyl	0.0	0.0			
Meloidogyne incognita					
Untreated	7.52	± 0.82	0.95	0.85	0.78
TOD	7.75	± 0.82			
Ethanol	7.46	± 0.66			
Oxamyl	0.0	0.0			
Aldicarb	0.0	0.0			

[a] Total number of nematodes per gram of root.
[b] SE = standard error
[c] Contrast of *P*-values
[d] U = untreated
[e] T = TOD
[f] E = ethanol

Table 10. Effect of TOD on the development of *Rotylenchulus reniformis* and *Meloidogyne incognita* on cotton roots. Means and standard errors (SE) are given as log(counts).

	Stage A[ab] (Mean ± SE[d])	Stage B[ab] (Mean ± SE)	Comparison (Pr>t)[c]					
			Stage A			Stage B		
			U[e] vsT[f]	U vs E[g]	T vs E	U vs T	U vs E	TvsE
Rotylenchulus reniformis								
Untreated	3.52 ± 0.13	2.90 ± 0.13	0.55	0.48	0.77	0.04	0.02	0.99
TOD	3.36 ± 0.21	3.26 ± 0.15						
Ethanol	3.43 ± 0.15	3.26 ± 0.12						
Meloidogyne incognita								
Untreated	2.06 ± 0.10		0.89	0.83	0.92			
TOD	2.09 ± 0.11							
Ethanol	2.08 ± 0.09							

[a] *R. reniformis*-nematode classification: Stage A: vermiform, non-swollen adult; Stage B: female body in open C, swollen shape in part of vulva (Diaz et al., 2003).
[b] *M. incognita*-nematode classification: Stage A: vermiform J2; Stage B: sausage-shaped J2 having a conical tail (Diaz et al., 2003).
[c] Contrast of *P*-values
[d] SE = standard error
[e] U = untreated
[f] T = TOD
[g] E = ethanol

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