Aminoglycosides and Syringomycin E as Fungicides Against
Fusarium graminearum in Head Blight Disease

Yukie Kawasaki
Utah State University

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AMINOGLYCOSIDES AND SYRINGOMYCIN E AS FUNGICIDES AGAINST

FUSARIUM GRAMINEARUM IN HEAD BLIGHT DISEASE

by

Yukie Kawasaki

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

BIOLOGY

Approved:

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Jon Y. Takemoto               Kent Evans
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UTAH STATE UNIVERSITY
Logan, Utah

2008
ABSTRACT

Aminoglycosides and Syringomycin E as Fungicides Against

*Fusarium graminearum* in Head Blight Disease

by

Yukie Kawasaki, Master of Science

Utah State University, 2008

Co-Major Professors: Dr. Jon Y. Takemoto and Dr. Kent Evans
Department: Biology

*Fusarium graminearum* (telemorph: *Gibberella zeae*) is one of the most problematic phytopathogens in US agriculture. This fungus causes head blight, foot rot, and damping off on wheat and barley. The infection lowers the grain yield and causes contamination of the grain product with mycotoxins. Effective control measures are lacking, and new fungicides that kill *F. graminearum* but remain safe and economical to use are needed. Newly synthesized aminoglycosides (JL22, JL38, JL39, JL40, NEOF004, NEOF005), classic aminoglycosides (amikacin, gentamicin, kanamycin A, kanamycin B, neomycin, and ribostamycin), and a lipopeptide, syringomycin E (SRE), were studied to determine their antifungal potential to control *F. graminearum*.

Aminoglycosides are protein synthesis inhibitors that mainly target bacteria, but a few were recently observed to kill fungi. They consist of an aminocyclitol ring bound with two or more amino sugars. Novel aminoglycosides were recently synthesized using novel glycodiversification synthetic schemes involving the replacement of the original amino sugars with unusual amino sugars.
SRE is an antifungal lipodepsinonapeptide produced by *Pseudomonas syringae* pv. *syringae*. This bacterium is an opportunistic pathogen in a wide range of plant species and produces several fungicidal lipopeptides. SRE forms pores on fungal plasma membrane and causes ion fluxes. An enhancement of its antifungal activity is reported in the presence of rhamnolipid surfactants.

The antifungal activities of various aminoglycosides, SRE, and a SRE-rhamnolipids mixture (SYRA) were determined against *F. graminearum* by measuring *in vitro* minimum inhibition concentrations (MICs) and *in planta* lesion area and chlorosis development using a leaf infection assay protocol. It was determined that using Tween® 20 at 0.2 % (v/v) concentration in the leaf infection assay promotes lesion development by *F. graminearum* with minimum phytotoxicity. *In vitro*, SRE, SYRA, and synthetic aminoglycoside JL38 showed the best antifungal activities. With the *in planta* assay, all three antifungal agents prevented infection by *F. graminearum*. However, inconsistent phytotoxicities were observed with SRE and SYRA that were influenced by the Tween® 20 surfactant included in the leaf infection assay. How Tween® 20 induces these phytotoxic inconsistencies is not known.

(67 pages)
I would like to thank Dr. Jon Takemoto for his great support, time, advice, kindness, and everything. He gave me the very first step into the depth of science and taught how fun research is.

I would like to thank Dr. Kent Evans for his guidance especially in leaf infection assay protocols and in general ideas about plant pathology.

I would like to thank Dr. Tom Chang for providing aminoglycoside compounds and his great knowledge in my research.

I would like to thank my lab mates; Mrs. Lynnette Takemoto for her support in both my study and my life, Dr. Michelle Grilley for her great help in research, Mekki Bensaci for his advice on graduate studies and research as a senior student, Sanjib Shrethsa for being a great fellow in struggling with graduate studies, and Matt Chambers and Nathaly Carranza for their help.

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Yukie Kawasaki
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CHAPTER 1
INTRODUCTION

Background

*Fusarium graminearum* and its diseases on cereal plants

*Fusarium avenaceum* (telemorph: *Gibberella avenaceum*), *F. culmorum* and *F. graminearum* (telemorph: *G. zeae*) are common pathogens on cereal plants in the world (30). In the United States, *F. graminearum* is prevalent and problematic on wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). It often exists as macroconidia (Figure 1-1) and causes head blight (scab), reduction in both quality and quantity of seed, bleaching, foot rot, and damping off. *F. graminearum* may infect other plant species without symptom development (15). Humidity and moderate temperatures (24-27 °C) accelerate disease development especially when host plants are in anthesis and having open florets.

On wheat plants, primary infection by *F. graminearum* occurs in the spring season. The fungus overwinters on plant debris by producing chlamidospores and asexual conidia (15, 30). When the temperature rises, maturations of perithecia follow production of ascospores and production of asexual conidia. Those inocula are dispersed by wind, rain-splash, irrigation system, and then infect host plants. Water-soaked brownish spots on leaves, stems, and grains are the first symptoms of infection. The lesions will spread in all directions from the point of infection and pink to purple fungal growth may be visible (16).

The production of mycotoxins, like the trichothecene deoxynivalenol (Figure 1-2)
and zearalenone (Figure 1-3), is a major problem resulting from \emph{F. graminearum} infections. Deoxynivalenol is an inhibitor of ribosomal protein synthesis in eukaryotic cells and is involved in the development of disease symptoms on plants (14). It is also known as vomitoxin which causes feed refusal and diarrhea when the contaminated grains are used as feed for livestock (4, 8, 31). Zearalenone is an estrogen mimic and causes volvovaginitis, pseudopregnancy, and infertility, in farm animals at high concentrations (4, 8, 33).

\emph{Fusarium} head blight is ranked as the worst plant disease by the United States Department of Agriculture (43). In the 1990s, there were several epidemics of \emph{Fusarium} head blight in the United States, and the economic losses were estimated at close to $3 billion (42). There have been efforts to improve chemical pesticides and genetic resistance to combat the disease, but these efforts have not succeeded. Currently, \emph{Fusarium} diseases are controlled by removal of crop residues, crop rotation, planting seeds free of \emph{Fusarium} inocula, and cultivation of relatively resistant varieties (5). However, crop rotation is limited in cereal-intensive production areas. The incidence and severity of \emph{Fusarium} head blight increases when the sowing of wheat or barley follows a corn crop. Moreover, the recent adoption of minimum tillage practices increases local sources of inocula and contributes to increased disease incidences (9).

\textbf{In planta assays of \emph{Fusarium} diseases}

To develop antifungal agents and resistant cultivars against \emph{Fusarium} species, a reliable and quick \textit{in planta} testing method is required. Hill (17) first described a foliar assay for characterizing reactions of wheat leaves as an indicator of resistance against \emph{F.}
culmorum, a root infecting pathogen that primarily causes dryland foot rot in cereals (35). Evans (11-13) modified Hill’s leaf assay protocol for use with *F. graminearum* and improved upon it for the study of early infection parameters of host-pathogen interactions on the foliar part. Even though the grain is the economically important portion of cereal plants, the leaf is preferred for such assays. Leaves are large enough for experimental handling a few weeks after seeding, and measurements of infection are easier on the flat leaf surface.

Briefly, the protocol involves: 1) inoculating *F. graminearum* on the primary leaves of wheat seedlings using the surfactant Tween® 20 (polyoxyethylene sorbitan monolaurate 20) and agar to increase adherence, 2) incubating plants under the optimum conditions for disease development, and 3) measuring induced lesion areas and relative chlorophyll content. This protocol is good for testing wheat cultivars for reaction to infection by *F. graminearum*, pathogenicities of *F. graminearum* isolates, and evaluating the effectiveness of antifungal agents.

**Current fungicides for controlling *F. graminearum***

It is reported that strobilurin and triazole fungicides have the ability to reduce disease symptoms caused by *Fusarium* species on wheat (5). There are three main problems in the usage of these chemical fungicides (21). The first difficulty is in their applications. To be effective, these reagents should be applied frequently and with proper timing, such as at or just after the beginning of anthesis. Second, frequent fungicide application is expensive and reduces the profit margin of the crop. The third problem is the doubt about the real or perceived safety of chemically treated crops for
animal or human consumption.

**Possible fungicides for controlling *F. graminearum***

1) **Aminoglycosides**

Aminoglycosides are naturally synthesized by *Actinomycetes*, and many aminoglycosides such as streptomycin, kanamycin, and neomycin are widely used as medical antibiotics. Aminoglycosides enter prokaryotic cells by active transport systems and bind to 30S subunits of ribosomes (24, 38). They cause errors in protein translation in target cells, and the resulting mis-translated and mis-folded proteins compromise cellular integrity (6). It was recently reported that certain commercially available aminoglycosides are inhibitory to plant pathogenic oomycetes (25). The antifungal modes of action of these aminoglycosides, however, are not known.

Aminoglycosides are compounds which have two or more amino sugars bound to an aminocyclitol ring through glycosidic bonds (24). Dr. C.-W. Tom Chang and his coworkers (Department of Chemistry and Biochemistry, Utah State University) are producing new aminoglycosides by synthetically combining different aminosugars and aglycons (10, 41). One of these, JL38 (an analog of kanamycin B) shows fungicidal but not antibacterial activities (unpublished, personal communication with Dr. Chang).

2) **Syringomycin E (SRE)**

Syringomycin E (SRE) is a lipodepsinonapeptide produced by certain strains of *Pseudomonas syringae* pv. *syringae*. This bacterium is an epiphyte that can be an opportunistic plant pathogen (2, 26, 28, 32). Like all natural product lipodepsipeptides,
SRE is synthesized by non-ribosomal peptide synthetases (1, 3, 34). SRE consists of 2-hydroxydodecanoate and a cyclic peptide composed of nine amino acids (Figure 4-1). It binds to the plasma membranes of target fungal cells, and forms pores for passive transmembrane ion fluxes (19, 20, 22, 27). Fungi and yeasts are sensitive, and bacteria are less susceptible or resistant to SRE (39). Although syringomycin is often described as a “phytotoxin” (1, 29, 39), the current view is that SRE instead has a role in fungal antagonism by *P. syringae* pv. *syringae* on plant surfaces (18, 23, 40).

SRE is lethal to many fungi including *Aspergillus* and *Fusarium* species (7, 36, 37). *In vitro*, it is fungicidal at concentrations as low as 0.1 μg/ml. In addition, M. Bensaci (Department of Biology, Utah State University) recently found that SRE mixed with rhamnolipids (Zonix™, Jeneil Biosurfactant, Co., Saukville, WI) increases its fungicidal activities. If the concentration of SRE that is lethal to fungi is not toxic to plants, it can be a useful natural product fungicide.

Goals of the Research

The main goal of this research was to test the antifungal activities of both pre-existing and newly synthesized aminoglycosides, SRE, and SRE mixed with rhamnolipids against *F. graminearum* both *in vitro* and *in vivo*. The *in vitro* activities were evaluated by determining the lowest concentrations of the aminoglycosides and SRE with and without rhamnolipids that were required to inhibit fungal growth. For the *in vivo* tests in wheat leaves, the Evans’ leaf assay protocol was used to test the fungal suppressive capabilities of these compounds.

Another aim of this research was to test the influence of varying concentrations of
Tween® 20 on the effectiveness of these compounds, host plant toxicity, and infection by *F. graminearum*. Tween® 20 is the standard surfactant used in the Evans’ leaf assay, but its influence on the assay has not yet been analyzed.

References


Figure 1-1. Macroconidial growth of *F. graminearum* in mung bean agar culture (200×).

Figure 1-2. Structure of deoxynivalenol.

Figure 1-3. Structure of zearalenone.
CHAPTER 2
INFLUENCE OF TWEEN® 20 ON LEAF INFECTION ASSAY
FOR FUSARIUM GRAMINEARUM

Introduction

The first known assay for infection by a Fusarium specie on leaves of wheat was devised by Hill (8). The inoculation was done with solidified agar disks containing ~35 conidia of *F. culmorum* placed on wounded leaves. Later, Evans modified this assay to make it easier, more reproducible, and reliable (2-4). The newer protocol uses less agar in solution and incorporates Tween® 20 (polyoxyethylene sorbitan monolaurate 20) to help infection of *F. graminearum*.

Because of their lack of ionization, non-ionic surfactants should be inert compared to cationic, anionic, and amphoteric surfactants (9). The primary purpose for using Tween® 20, a non-ionic surfactant, in this leaf infection assay, is to lower the surface tension of water. It serves as a wetting agent for inoculating *F. graminearum* on the hydrophobic leaf surface of wheat. Since surfactants increase the stomatal penetration of aqueous solutions into leaves (6), Tween® 20 may promote fungal penetration into stomata. An example of this latter effect was shown with severe disease development by *Pleospora papaveracea* with Tween® 20 on opium poppy (1).

Despite its positive attributes, undesirable effects of Tween® 20 are also possible. Negative effects of surfactants on plant cells reported with similar detergents are changes in permeability (7), inhibition of mitosis (9), and inhibition of photophosphorylation (10). Non-ionic surfactants can be interactive through the
hydrophobic properties and may contribute to errors in the leaf infection assay.

The main objective of the work described in this chapter is to determine the optimal concentration of Tween® 20 in the Evans’ leaf assay that induces fungal infection and with minimal damage to the host plant. Three concentrations of Tween® 20, 0.02, 0.08, and 0.2 % (v/v), were inoculated on the primary leaves of wheat with and without *F. graminearum*, and effects on fungal infection and phytotoxicities were observed. Tween® 20 was not phytotoxic at all tested concentrations and higher concentrations of the surfactant induced higher degree of fungal infection.

Materials and Methods

**F. graminearum strain and growth**

In the summer of 1995, *Fusarium* species were collected from cereal plants and identified by Dr. Kent Evans, working in the University of Minnesota Small Grains Pathology Program. One isolate of *F. graminearum*, strain B-4-5A, obtained from Minnesota, was selected for use in these experiments. To avoid production of mycotoxins, *F. graminearum* was grown on mung bean agar medium (5). Mung bean seeds (40 g/l) were boiled in water for 21 min. After removing the seeds, the broth was mixed with agar (15 g/l) and autoclaved for 30 min. After cooling (but not below 45 °C), the agar medium was poured into plastic petri plates. The pathogen was grown in the agar plates for 7 days under fluorescent lights at room temperature.

**Host plants**

Two cultivars of wheat, Alsen and Frontana, were used for this assay. Evans
reported that the cultivar Alsen is relatively susceptible and that the cultivar Frontana is relatively resistant to the leaf infection by *F. graminearum* (3, 4). Seeds were harvested from the parent plants maintained at the Small Grains Pathology Program at the University of Minnesota. They were stored at 4 °C after drying to break dormancy.

Leaf infection assay with different concentrations of Tween® 20

Seeds of Alsen and Frontana cultivars were planted in Cone-tainers (2.5 cm diameter, 16 cm length, Hummert International, Earth City, MO) packed with Sunshine #4 potting medium (Sun Gro Horticulture Inc., Bellevue, WA) and grown in a greenhouse at 20-23 °C. The plants reached the two-leaf stage in two weeks after seeding and they were moved to the lab for inoculation.

The experiment was designed with one control group and six treatment groups on both Alsen and Frontana cultivars with ten replications for each group. The control plants were not inoculated. Three of the six treatment groups were for testing phytotoxicity of Tween® 20; 10 μl of the surfactant was inoculated in the middle of the primary leaves at concentrations of 0.02, 0.08, and 0.2 % (v/v) in 0.25 % (w/v) agar solution. For fungal treatments, *F. graminearum* (1000 macroconidia) was inoculated in the same manner with the same three concentrations of Tween® 20 in 0.25 % (w/v) agar solution.

After the inocula dried on the leaves (1-2 h), the plants were placed in a dew chamber (24 °C, 12 h light/12 h dark, ~100% humidity) for 72 h to provide optimum infection developing conditions. The seedlings were then taken out of the chamber and set on a lab bench for 24 h at room temperature and ambient lighting.
Lesions by *F. graminearum* developed on the primary leaves of wheat after 96 h post-inoculation. First, the leaves were detached from the plants and the degree of infection was measured as percent lesion area (infected area / the area of a 3 cm length of primary leaf × 100 %) and relative chlorophyll content. Digital photographs of the leaves were taken to quantify the lesion area using APS Assess (plant disease quantification software, APS Press, St Paul, MN) and to calculate the percent lesion area. Then, the relative chlorophyll contents of inoculated areas were measured using a CCM-200 Chlorophyll Content Meter (OPTICSCIENCE, Tyngsboro, MA). The data were exported to a spreadsheet and analyzed by Statistix software (STATISTIX, Tallahassee, FL).

Results and Discussion

Four experiments were performed for testing the influences of Tween® 20 on host plants and fungal infection. Figure 2-1 shows a typical healthy leaf and an infected leaf. The leaves infected by *F. graminearum* showed yellow to brown chlorotic and necrotic lesion, and fungal growth was observed within the lesion areas under a light microscope (Figure 2-2). Severe lesions were observed on leaves of Alsen and few or no fungal infections were observed on Frontana leaves (Figure 2-3 and Figure 2-4). The degree of green color and leaf thickness were generally observed to be much higher in Frontana plants.

The inocula, 0.02, 0.08, and 0.2 % (v/v) of Tween® 20 without *F. graminearum*, did not induce significant lesions on leaves assessed for percent lesion area (Figure 2-3). The lesions developed under treatments with the pathogen were due to the fungal infection...
and not the surfactant. The degree of fungal infection increased as the Tween® 20 concentration increased in Alsen. \textit{F. graminearum} with 0.2 % (v/v) of the surfactant caused the largest lesion areas. At this concentration, significant decreases were always found in relative chlorophyll content values (Figure 2-4). The presence of Tween® 20 at 0.2 % (v/v) concentration accelerates lesion development by \textit{F. graminearum} with minimum phytotoxicity.

Several factors affect the results in this leaf infection assay. For Example, humidity was critical in 72 h post-incubation period because lesion development is hindered with low humidity. Another factor is plant health prior to inoculations especially in the summer when plants can get photooxidized and yellowed leaves under intense sunlight. Also, insects (e.g. aphids) are parasitic on wheat seedlings in the warmer seasons and promote transfer of pathogenic microbes. Such factors compromise the ability to make accurate measurements of fungal leaf infections.

References


Figure 2-1. Comparison of a non-infected leaf (A) and an infected leaf (B).

Figure 2-2. Growth of *F. graminearum* on the surface of an infected leaf (200×).
Figure 2-3. Percent lesion areas developed with inocula containing 0.02, 0.08, and 0.2 % (v/v) of Tween® 20. The lesion areas in four separate experiments were measured by digital photography and analysis with APS Assess (plant disease quantification software, APS Press, St Paul, MN). The diseased area by the infection of *F. graminearum* increased as the Tween® 20 concentration was increased in the inoculum. Tween® 20 induced significant degree of fungal infection on Alsen at 0.2 % (v/v) without phytotoxicity in three out of four experiments. There were no effects on Frontana.
3<sup>rd</sup> experiment

4<sup>th</sup> experiment
Figure 2-4. Relative chlorophyll contents in leaf infection assays with 0.02, 0.08, and 0.2 % (v/v) of Tween® 20. Results of four separate experiments measured using a CCM-200 Chlorophyll Content Meter (OPTICSCIENCE, Tyngsboro, MA) are shown. *F. graminearum* caused significant reduction in relative chlorophyll content value when inoculated with 0.2 % (v/v) Tween® 20.
3\textsuperscript{rd} experiment

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Relative chlorophyll content

4\textsuperscript{th} experiment

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Relative chlorophyll content
CHAPTER 3
AMINOGLYCOSIDES AS FUNGICIDES AGAINST
FUSARIUM GRAMINEARUM

Introduction

Aminoglycosides are aminocyclitol antibiotics consisting of an aminocyclitol ring bound to two or more amino sugars through glycosidic linkages (Figure 3-1) (15). Amino groups give aminoglycosides net positive charges and increase the affinity to negatively charged residues on the surfaces of bacterial cells. Aminoglycosides diffuse across the outer membrane into the periplasmic space by passive transport, and across the cytoplasmic membrane via active transport (1, 24, 25). Then, they cause mis-reading of mRNA by binding to the 30S subunit of rRNA in bacterial protein biosynthesis (4, 15). Mis-translated and mis-folded cell surface proteins disrupt the integrity of the bacterial cell wall. Because of this mechanism of action, aminoglycosides are effective against gram-negative and aerobic bacteria.

Since the discovery of streptomycin in 1944, aminoglycosides have been an effective solution in infectious disease treatments. As the usage of aminoglycosides increased, resistance among microbes also developed (1, 2, 15, 26). There are four aminoglycoside resistance mechanisms in bacteria (24). The first mechanism is deactivation of aminoglycosides by evolving enzymes capable of adenylating, acetylating, and phosphorylating the hydroxyl and amino groups (20). The second is the reduction of intracellular concentration of aminoglycosides by drug trapping, active efflux, and changes in membrane permeability. The third mechanism is prevention of
aminoglycoside binding by mutant defects in the target site of ribosomes. The last kind of resistance mechanism is methylation of the aminoglycoside binding site.

To counteract the development of resistances, numerous chemical modifications on existing aminoglycosides have been made to bypass the enzymatic activities of resistant bacteria and to restore antimicrobial activities (12, 16, 17, 19, 27). There exist many examples of aminoglycosides that show altered affinities to bacterial targets and lower activities when their functional groups are replaced (15). In the laboratory of Dr. C.-W. Tom Chang (Department of Chemistry and Biochemistry, Utah State University), new aminoglycosides are synthesized using novel glycodiversification synthetic schemes (5, 28). Naturally occurring aminoglycoside analogues were synthesized by replacing the original amino sugars with unusual amino sugars. JL22, JL38, JL39, and JL40 are synthesized as kanamycin B analogues, and NEOF004 and NEOF005 are neomycin analogues.

Aminoglycoside inhibition of fungi is not well known, but a recent report of fungicidal activities against Phytophthora and Pythium species has been published (18). The objective of the current research described in this chapter is to determine the antifungal activities of classical and novel synthetic aminoglycosides against Fusarium graminearum, the pathogen on cereal plants that causes yield loss and contamination of grains with mycotoxins (11, 22). Strobilurin and triazole fungicides are currently used (3, 14) but there is no complete success in the control of F. graminearum by these agents. Because an epidemic of this fungal disease can greatly impact grain production and the related economics (29), there is a clear need for effective control agents directed against F. graminearum.
Six newly synthesized aminoglycosides, JL22, JL38, JL39, JL40, NEOF004 and NEOF005, and six commercially available aminoglycosides, amikacin, gentamicin, kanamycin A, kanamycin B, neomycin, ribostamycin, were tested. In vitro antifungal activities were determined and quantitated as minimum inhibitory concentrations (MICs). The aminoglycosides observed to have lower MICs were tested in planta with the Evans’ leaf infection assay (6-8). Selected aminoglycosides were placed on the primary leaves of wheat with F. graminearum macroconidia to see their ability to prevent fungal infection. Among all aminoglycosides tested, JL38 showed the best fungicidal activities. JL38 and two more active aminoglycosides, JL40 and kanamycin B, were tested in planta. All three aminoglycosides showed phytotoxicity but only JL38 was able to suppress fungal infection at levels that do not damage plants.

Material and Methods

Aminoglycosides

All aminoglycosides were kindly provided by the laboratory of Dr. Tom Chang (Department of Chemistry and Biochemistry, Utah State University). They were stored as 10 mg/ml solutions in water at 4 °C.

Growth Medium

Fresh potato dextrose broth (PDB) + casamino acids was used throughout. To make 1 l of PDB + casamino acids, 200 g of fresh potatoes were boiled in 500 ml of distilled water for 30 min. The broth was filtered through 2 layers of cheese cloth, and the volume was brought up to 1 l. After additions of 20 g of glucose (2 %, w/v) and 4 g
of casamino acid (0.4 %, w/v), the mixture was stirred with a magnetic bar until all solids were dissolved. Then, the medium was sterilized by autoclaving for 30 min. Potato dextrose gar (PDA) + casamino acids medium was prepared with 15 % of agar and poured into plastic petri plates.

**Isolates of *F. graminearum***

Two isolates of *F. graminearum*, B-4-5A and Butte86ADA-11 were obtained from the Small Grain Pathology Program of the University of Minnesota. Both strains were used for MIC tests and only B-4-5A was used for leaf infection assays.

**MIC tests**

The MICs of 12 aminoglycosides, JL22, JL38, JL39, JL40, NEOF004, NEOF005, amikacin, gentamicin, kanamycin A, kanamycin B, neomycin, and ribostamycin, were estimated in sterile, flat-bottomed 96-well microtiter plates (Corning Costar, Corning, NY) in the range of 500 to 1 µg/ml. Stock solutions were prepared at a concentration of 2 mg/ml in water. In a 96-well plate, 50 µl aliquots of each aminoglycoside stock solution was added in the column 3 wells and two-fold serial dilutions were made 10 times (to column 12) with sterile distilled water. Then, 40 µl of PDB + casamino acids and 10 µl of $10^5$ macroconidia/ml *F. graminearum* suspension were added to the wells. Negative (90 µl of PDB + casamino acids and 10 µl of sterile distilled water) and positive (90 µl of PDB + casamino acids and 10 µl of $10^5$ macroconidia /ml of *F. graminearum*) controls were placed in wells of columns 1 and 2, respectively. The plates were incubated at 24 °C for 72 h. The optical densities of the wells were measured using an ELx800 Absorbance Microplate Reader (BioTek Instruments Inc., Winooski, VA) with a
wavelength of 630 nm every 12 h after inoculation. The percentages of inhibition of fungal growth were calculated as follows: \[
\frac{(\text{A630 of a well at 72 h}) - (\text{A630 of a well at 0 h})}{(\text{A630 of column 2 at 72h}) - (\text{A630 of column 2 at 0 h})} \times 100\%
\]. MIC tests were replicated four times and each treatment was repeated at least two times.

The *in planta* experiments use Tween® 20 as an adjuvant, and there are possibilities that this surfactant enhances, suppresses, or eliminates the activities of aminoglycosides (10, 13, 21). To test these effects of Tween® 20, MIC tests were done in the presence of Tween® 20. Aminoglycosides that showed activities against *F. graminearum* were tested with PDB + casamino acids and Tween® 20 surfactant. Tween® 20 was mixed into PDB + casamino acids at concentrations of 0.05, 0.2, or 0.5 % (v/v). Forty µl portions of these media were added to the microtiter plate wells to give 100 µl final volumes of *F. graminearum*–containing suspensions (10⁴ macroconidia/ml) under aminoglycoside treatments with final concentrations of Tween® 20 of 0.02, 0.08, or 0.2 % (v/v). The incubation and growth measurement procedures were the same as MIC tests without Tween® 20.

**Leaf infection assay**

JL38, JL40, and kanamycin B were tested for their antifungal abilities *in planta*. Because the amounts of those reagents were limited, they were applied by mixing in inocula rather than spraying the entire plants. Solutions containing aminoglycosides at 30 µg/ml, 180 µg/ml, and 1080 µg/ml in 0.25 % (w/v) agar and 0.2 % (v/v) Tween® 20 were prepared to test their phytotoxicities. The pathogen was grown on mung bean agar plates (9) for 7 days, and the suspension (2.0×10⁴ macroconidia/ml) was prepared in
sterile 0.25 % (w/v) agar solution with 0.2 % (v/v) of Tween® 20. It was mixed 1:1 by volume with solutions containing either 60 μg/ml, 360 μg/ml or 2160 μg/ml of aminoglycoside in 0.25 % (w/v) agar solution containing 0.2 % (v/v) Tween® 20. Final inocula and aminoglycoside concentrations were 10⁴ macroconidia/ml and 30 μg/ml, 180 μg/ml or 1080 μg/ml, respectively. Non-treated, negative control plants, and positive control plants inoculated with 10⁴ macroconidia/ml in 0.25 % (w/v) agar and 0.2 % (v/v) Tween® 20 solution, respectively, were also prepared. Inoculation procedures, incubation conditions, and data collections were as described in Chapter 2. Eight replications were performed for each treatment for both Alsen and Frontana cultivars. Each whole set of experiments was repeated three times.

After the measurements, the inoculated parts of the leaves were cut out as 5 mm length segments. The surfaces of the leaf segments were rinsed with 30 % bleach and sterile distilled water two times, and placed on surfaces of PDA + casamino acid agar plates (15 % w/v agar). The plates were incubated at 24 °C for 48 h and fungal growth from the segments determined by visual observation.

Results and Discussion

**In vitro MIC tests**

The concentrations of each aminoglycoside required to inhibit 90 % of the growth or more (MIC<sub>90</sub>) are summarized in Table 3-1. Amikacin, JL22, JL39, kanamycin A, and ribostamycin were not lethal to *F. graminearum* in the range of 500 to 1 μg/ml. Gentamicin, JL40, kanamycin B, NEOF004, NEOF005, and neomycin were fungicidal in the range of 250 to 62.5 μg/ml. The most active anti-*Fusarium* aminoglycoside tested
was JL38 with an MIC of 31.3 μg/ml (Figure 3-2).

The structural difference between kanamycin A and kanamycin B is the occurrence of a hydroxyl or amino group at 2’ position of ring I, respectively. This difference may account for the difference in their antifungal activities. New aminoglycosides, JL38 and JL40, are kanamycin B derivatives which have different amino sugars in the ring III. Both have increased antifungal activity compared to kanamycin B (Table 3-1). These two synthetic analogs represent successful improvement of antimicrobial activity of an aminoglycoside by glycodiversification.

JL38, JL40, and kanamycin B were selected for further testing. The MICs of these three drugs were determined in PDB + casamino acids medium containing 0.02, 0.08 or 0.2 % (v/v) Tween® 20. Tween® 20 did not affect the in vitro activities of all three aminoglycosides (Table 3-1). NEOF004 and NEOF005 had the same or better activities compared to JL40 and kanamycin B. However, they were not tested further because of the limited resources (space and materials) for more in planta experiments.

**In planta leaf infection assay**

JL38, JL40, and kanamycin B were tested for their in planta antifungal activities against *F. graminearum* isolate B-4-5A (Figures 3-3 and 3-4). Although JL38, JL40, and kanamycin B showed activities in vitro, only JL38 showed in vivo fungicidal activities at concentrations that were also non-phytotoxic. For example, JL38 at 30 μg/ml prevented lesion development by *F. graminearum* while having no phytotoxicity.

While JL38 was selectively fungicidal at 30 μg/ml, it was phytotoxic at higher concentrations since the degree of chlorosis was severer compared to controls. Because
there was no fungal growth from the leaf segments treated with higher concentrations of JL38 (Figure 3-5), chlorosis was caused by JL38 and not *F. graminearum*. Additionally, the Frontana cultivar was relatively resistant to both infection by *F. graminearum* and JL38 phytotoxicity as compared to the Alsen cultivar.

In contrast to JL38, JL40 and kanamycin B were phytotoxic at 30 μg/ml (Figures 3-3 and 3-4). Leaf segments treated with JL40 and kanamycin B at this concentration showed mycelial growth of *F. graminearum* (Figure 3-6 and 3-7), and macroconidia characteristic of *Fusarium* species were observed microscopically (Figure 3-8). Therefore, at this concentration, JL40 and kanamycin B did not prevent leaf infection by *F. graminearum*.

In summary, JL38 controlled both *in vitro* and *in vivo* growth of *F. graminearum*, while JL40 and kanamycin B were effective only *in vitro* at higher concentrations. JL38 has fungicidal but no antibacterial activities (unpublished, personal communication, M. Bensaci, Biology, Utah State University). It is structurally different from other kanamycin B analogs due to the presence of a carbon alkyl chain on ring III. This structural feature might function in promoting JL38’s antifungal activity which the parent molecule kanamycin B lacks. A fungal specific aminoglycoside drug such as JL38 will be beneficial in crop protection strategies because it would likely not promote bacterial resistance as do many conventional aminoglycosides (23).

References


14. **Jones, R. K.** 2000. Assessments of *Fusarium* head blight of wheat and barley in
response to fungicide treatment. Plant Dis. **84**:1021-1030.


Figure 3-1. Structures of aminocyclitols. Aminoglycosides are aminocyclitol antibiotics consisting of an aminocyclitol ring (shown above or their derivatives) bound with amino sugars through glycosidic bonds.
Figure 3-2. MIC determination of JL38 against *F. graminearum*. Column 1: negative control (PDB + casamino acids), column 2: positive control (PDB + casamino acids and $10^4 F. graminearum$ macrocinidia), and columns 3 to 12: 2-fold dilutions of JL38 (500-1 µg/ml in PDB + casamino acids and $10^4 F. graminearum$ macroconidia). Rows A to D were tests against B-4-5A and E to H were against Butte86ADA-11. The photograph was taken after 72 h incubation at 24 °C. Compared to the positive control, more than 90% of the growth was inhibited in columns 3 to 7, and there was no visible growth. In columns 8, 9, and 10, the fungal growth was partially inhibited and the color was creamy white to yellow. Columns 11 and 12 were yellow to red and the growth was not suppressed.
### Table 3-1. Estimated MICs of aminoglycosides against *F. graminearum*

<table>
<thead>
<tr>
<th></th>
<th>AMIKACIN</th>
<th>GENTAMICIN</th>
<th>JL22</th>
<th>JL38</th>
<th>JL39</th>
<th>JL40</th>
<th>KANAMYCIN A</th>
<th>KANAMYCIN B</th>
<th>NEOF004</th>
<th>NEOF005</th>
<th>Neomycin</th>
<th>Ribostamycin</th>
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<td>Butte86ADA-11</td>
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Figure 3-3. Percent lesion areas developed in leaf infection assays with JL38, JL40 and kanamycin B treatments. The lesion areas were measured by digital photography and analysis with APS Assess (plant disease quantification software, APS Press, St Paul, MN). Results of three separate experiments are shown. Aminoglycosides were applied to wheat seedlings at 30, 180, and 1080 μg/ml (in 0.25 % [w/v] agar solution including 0.2 % [v/v] Tween® 20) with and without F. graminearum macroconidia. Thirty μg/ml of JL38 prevented the lesion area developments while having no phytotoxicities.
Figure 3-4. Relative chlorophyll contents in leaf infection assays with JL38, JL40 and kanamycin B treatment. Results of three separate experiments measured using a CCM-200 Chlorophyll Content Meter (OPTICSCIENCE, Tyngsboro, MA) are shown. Aminoglycosides were applied to wheat seedlings at 30, 180, and 1080 μg/ml (in 0.25 % [w/v] agar solution including 0.2 % [v/v] Tween® 20) with and without *F. graminearum*. JL38 showed no chlorophyll damages at 30 μg/ml while it became phytotoxic at higher concentrations. JL40 and kanamycin B were phytotoxic at all tested concentrations.
2\textsuperscript{nd} experiment

![Graph showing relative chlorophyll content for two cultivars (Alsen and Frontana) across different treatments.]

3\textsuperscript{rd} experiment

![Graph showing relative chlorophyll content for two cultivars (Alsen and Frontana) across different treatments.]

Cultivar
Alsen
Frontana
Figure 3-5. Growth from leaf segments with JL38 treatments. Leaf segments of Alsen cultivar treated with JL38 were plated on PDA + casamino acids plates and incubated at 24 °C for 48 h. A: negative control (no inoculation), B: positive control (F. graminearum), C: 30 µg/ml, D: 30 µg/ml + F. graminearum, E: 180 µg/ml, F: 180 µg/ml + F. graminearum, G: 1080 µg/ml, H: 1080 µg/ml + F. graminearum. No fungal growth was observed from the leaf segments treated with JL38.
Figure 3-6. Growth from leaf segments with JL40 treatments. Leaf segments of Alsen cultivar with JL40 treatments were plated on PDA + casamino acids plates and incubated at 24 °C for 48 h. A: negative control (no inoculation), B: positive control (*F. graminearum*), C: 30 µg/ml, D: 30 µg/ml + *F. graminearum*, E: 180 µg/ml, F: 180 µg/ml + *F. graminearum*, G: 1080 µg/ml, H: 1080 µg/ml + *F. graminearum*. JL40 did not control the infection of *F. graminearum* at all tested concentrations.
Figure 3-7. Growth from leaf segments with kanamycin B treatments. Leaf segments of Alsen cultivar treated with kanamycin B were plated on PDA + casamino acids plates and incubated at 24 °C for 48 h. A: negative control (no inoculation), B: positive control (F. graminearum), C: 30 µg/ml, D: 30 µg/ml + F. graminearum, E: 180 µg/ml, F: 180 µg/ml + F. graminearum, G: 1080 µg/ml, H: 1080 µg/ml + F. graminearum. Kanamycin B did not control the infection of F. graminearum at all tested concentrations.
Figure 3-8. Macroconidial growth from a leaf segment treated with JL40. Macroconidia, characteristic of *F. graminearum*, were observed in the growth from leaf segments treated with 180 μg/ml of JL40 (200×).
CHAPTER 4
SYRINGOMYCIN E AS A FUNGICIDE AGAINST
FUSARIUM GRAMINEARUM

Introduction

Bacteria often secrete toxins to build a suitable environment for their survival and growth (11). *Pseudomonas syringae* pv. *syringae* is an epiphyte on many woody and herbaceous plants. This bacterium can also be an opportunistic plant pathogen involved in blights, leaf spots, and galls. Various ecotypes of *P. syringae* pv. *syringae* strains produce two classes of bioactive cyclic lipodepsipeptides commonly known as syringomycins and syringopeptins (17) via non-ribosomal peptide biosynthesis (1, 4, 15). Syringomycins are small (MW = 1100-1300) lipodepsipeptides with potent antifungal activities (8, 17), and syringopeptins are larger lipodepsipeptides (MW = 2200-2500) that cause plant tissue necrosis. Syringomycin E (SRE) is a major form of the syringomycins. It consists of 2-hydroxydodecanoate (non-polar tail) and a cyclic peptide composed of nine amino acids (polar head) (Figure 4-1). SRE inserts into the target membranes of fungi by its amphiphilic property and forms pores allowing for passive mono- and bivalent ion fluxes (7, 8, 10, 13, 16, 17). *Geotrichum candidum*, *Rhodototula pilimanae*, and *Saccharomyces cerevisiae* are examples of fungi and yeasts reported to be extremely sensitive to SRE (16).

Rhamnolipids (Figure 4-2) are rhamnose-containing molecules biosynthesized by *P. aeruginosa* strains (5, 9, 14). Rhamnolipids disrupt the membranes of zoospore fungi at relatively high concentrations (12). In the zoospore stage, fungi have no cell wall and
the damage on the cell plasma membrane by surfactants can be critical. On the other hand, fungal cells are resistant to rhamnolipids when they are in stages where cell walls are present (such as the macroconidial stage of *F. graminearum*).

Both SRE and rhamnolipids are amphiphilic, but their antifungal spectra and mechanisms of action are different. M. Bensaci (Department of Biology, Utah State University) recently found that the fungicidal activity of SRE increases when it is mixed with rhamnolipids (Zonix™, Jeneil Biosurfactant Co., Saukville, WI) (2). The mechanisms by which rhamnolipids interact with SRE are not clear, but rhamnolipids can lower the MICs of SRE 2 to 4 times against a broad range of fungi including *Fusarium*, *Aspergillus*, and *Saccharomyces*.

The objective of the research described in this chapter is to determine if SRE and the SRE-rhamnolipid mixtures will inhibit *F. graminearum* and suppress its infection of wheat seedlings. SRE was produced and purified by methods similar to those described by Bidwai et al. (3). *In vitro* MIC determinations and *in planta* leaf infection assays for SRE and a SRE-rhamnolipid mixture (SRE : rhamnolipids = 1 : 3 [w/w]) named SYRA were performed as described for the aminoglycosides in Chapter 3 of this thesis. SRE and SYRA showed low MICs ranging between 31.3 and 7.8 µg/ml, and 15.6 and 7.8 µg/ml against two strains of *F. graminearum*. They were also active *in planta* at concentrations as low as 5 µg/ml, but phytotoxicities were also observed at this concentration when the surfactant Tween® 20 was included.
Purification of syringomycin E

Growth of *P. syringae* pv. *syringae* B301D for syringomycin production

*Pseudomonas syringae* pv. *syringae* strain B301D was grown to produce syringomycin E. The strain is maintained in the laboratory of J. Takemoto (Department of Biology, Utah State University) on potato dextrose agar (PDA) medium containing 4 % (w/v) casamino acids. Prior to the experiments, it was inoculated into five Kimax culture tubes each containing 5ml of potato dextrose broth (PDB) + casamino acids medium. The cultures were grown with rotary shaking at 28 °C for 24 h. Sixteen 500 ml-capacity Erlenmeyer flasks were each filled with 250 ml of PDB + casamino acids medium and inoculated with 1 ml of the actively growing culture. The flask cultures were incubated at 28 °C without shaking. Seven to 10 days of growth are typically required to reach maximum production of syringomycin.

Bioassay of syringomycin

Bioassays of syringomycin were done by spotting 8 µl aliquots of culture extracts onto a freshly spread lawn of *Rhodotorula pilimanae* on PDA + casamino acids medium. From the growing culture, 500 µl of sample was taken into a microcentrifuge tube and mixed with 500 µl of acidified acetone (0.4 % HCl in acetone). The tube was centrifuged at 10,000 rpm for 3 min, and 200 µl of the supernatant fluid was removed into a well of a Corning ceramic plate. The plate was heated at about 45 °C to evaporate the residue into a small oil. The residue was resuspended in 100 µl of sterile
distilled water and 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64 dilutions were made. The surface of a PDA + casamino acids medium plate was streaked with *R. pilimanae* by using a cotton swab. Each dilution was spotted onto the *R. pilimanae* lawn in volumes of 8 µl. After 24 h incubation at room temperature, cleared zones of growth inhibition were observed with extracts containing syringomycin. To continue with syringomycin E purification (see below), inhibitory activity must be observed with at least 1/8 dilution of the extract.

**Syringomycin E purification**

The culture flasks were chilled to 4 °C and mixed with acidified acetone. The cell debris was removed by centrifugation (Beckman JA10 rotor, 7,000 rpm, 20 min). The supernatant was concentrated to 10 % of the initial culture volume by rotary flash evaporation at 45 °C. The volume was adjusted with acetone to make final concentration of acetone 60 %. The suspension was left stirring gently overnight at 4 °C. Then, it was centrifuged again to remove any solid phase materials, and acetone was removed by rotate evaporation. The final volume was adjusted to 1.5 l with distilled water. An Amberlite XAD-2 (20 to 50 meshes) column (3 × 25 cm) was used for the first step of purification. After washing successively with methanol, iso-propanol, and 0.1 % trifluoroacetic acid (TFA) in water, the column was equilibrated with 0.1 % TFA. The sample was dripped onto the column at a rate of 1-3 ml/min. Then, syringomycin was eluted with 0.1 % TFA using a non-linear gradient of iso-propanol in 0.1 % TFA over a 360 min cycle. The proportions of iso-propanol in the gradient were as follows: 0 to 20 % (linear) for 20 min, constantly 20 % for 120 min, 20
to 70 % (linear) for 140 min, constantly at 70 % for 30 min, 70 to 100 % (linear) for 30 min, and finally constantly at 100 % for 60 min. Fractions were collected separately every 4 min. After testing each fraction for antifungal activity against *R. pilimanae*, the active fractions were pooled. The pooled fractions were rotary evaporated, lyophilized, and dissolved in 60 % acetone in water. This solution was subjected to high performance liquid chromatography on a 1 × 25 cm, C18 silica reverse phase column with a linear gradient of 0-100 % solvent B in 45 min (solvent A, 0.1 % TFA and solvent B, 0.1 % TFA in iso-propanol). The SRE peak was identified by its characteristic R<sub>f</sub> value of 21 min and activity by bioassay with *R. pilimanae*. SRE peak materials from multiple chromatographic runs were collected, pooled, and lyophilized.

**Preparation of SYRA**

Rhamnolipids used were a commercial source (Zonix™ biofungicide, Jeneil Biosurfactant Co., Saukville, WI). The purified SRE were mixed with rhamnolipids in a 1 : 3 (SRE : rhamnolipid, [w/w]) ratio to make SYRA.

**MIC tests**

The MICs of SRE, SYRA, and rhamnolipids were measured using the same methods described in Chapter 3. The testing ranges were 125-0.25 μg/ml for SRE and SRE in SYRA, and 500-1.0 μg/ml for rhamnolipid.

**Leaf infection assay**

Syringomycin E and SYRA were applied for leaf infection assay at concentrations of 5, 30, 180 μg/ml in the same manner as described in Chapter 3.
Results and Discussion

Purification of syringomycin E

From 4 l of cultured *P. syringae* pv. *syringae* strain B301D, 80 mg of chromatographically pure syringomycin E was obtained. It was stored in powder form at -20 °C until use in the MIC determination tests and in the leaf infection assays. The SRE-rhamnolipid mixture, SYRA, was prepared as 500 µg/ml of SRE and 1500 µg/ml of rhamnolipids.

MIC tests

The estimated MICs for SRE and SYRA against *F. graminearum* are shown in Table 4-1. The MICs of SRE ranged between 31.3 and 7.8 µg/ml against two isolates of *F. graminearum*, B-4-5A and Butte86ADA-11. In the absence of Tween® 20, isolate B-4-5A was less susceptible to SRE compared to isolate Butte86ADA-11. When mixed with three times by weight of rhamnolipids (i.e. in SYRA), the MIC value for the SRE component of SYRA decreased 2-fold against B-4-5A. The rhamnolipids alone were not fungicidal against *F. graminearum* (MIC > 500 µg/ml). Tween® 20 also lowered the MIC of SRE against B-4-5A. Mixing with rhamnolipids and Tween® 20 dramatically decreased the *in vitro* fungicidal activity of SRE (Table 4-1). However, SYRA still prevented pigment production of *F. graminearum* at 3.8 µg/ml, as SRE did at 1.9 µg/ml in the presence of Tween® 20 (Figure 4-3). The characteristic yellow, red, and violet colors of *Fusarium* cultures come from the production of mycotoxins, naphoquinones (6). The suppression of mycotoxin production possibly reduces disease developments by *F. graminearum*. 


Leaf infection assays

Figure 4-4 and Figure 4-5 show the results of leaf infection assays with treatments of SRE and SYRA. In three separate experiments, the results were variable. In the first experiment, the phytotoxicities of SRE and SYRA were strong and no indications for disease control were observed in percent lesion areas and relative chlorophyll contents because of the phytotoxic effects (Figures 4-4 and 4-5). However, less phytotoxicity with SRE and SYRA was observed in the second and the third experiments (Figures 4-4 and 4-5). In these latter experiments, SYRA lowered the disease symptoms with respect to both percent lesion areas and relative chlorophyll contents. With treatments of SRE and SYRA no growth of *F. graminearum* was observed from the treated leaf segments (Figure 4-6 and 4-7). SYRA prevented fungal infection on leaves although it was not fungicidal in the MIC determination test. The activity of SYRA observed against *F. graminearum in vitro* was suppression of pigment mycotoxin production (Figure 4-3). Pigmented mycotoxins possibly have important roles in invasion of phytophagic fungi into the plants.

In summary, SRE and SYRA prevented fungal infection of *F. graminearum* but showed phytotoxicities when they were applied with Tween® 20. The effective concentrations of these agents and degree of their phytotoxicities were not consistent among three experiments. Complex relationships between SRE, Tween® 20, rhamnolipids, and plants might exist, and these were perhaps not controlled in the presently described experiments. For SRE to be used in the field to control wheat head blight, the mechanisms of how it interacts with surfactants such as Tween® 20 and the influences of such interactions on antifungal and phytotoxic activities must be clarified.
References


8. **Iacobellis, N. S., P. Lavermicocca, I. Grgurina, M. Simmaco, and A. Ballio.**


Figure 4-1. Structure of syringomycin E.

Figure 4-2. Structure of rhamnolipids.
### Table 4-1. Estimated MICs of rhamnolipids, SRE, and SYRA against *F. graminearum*

<table>
<thead>
<tr>
<th></th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (µg/ml)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 % (v/v) Tween&lt;sub&gt;20&lt;/sub&gt;</td>
<td>0.02 % (v/v) Tween&lt;sub&gt;20&lt;/sub&gt;</td>
<td>0.08 % (v/v) Tween&lt;sub&gt;20&lt;/sub&gt;</td>
<td>0.2 % (v/v) Tween&lt;sub&gt;20&lt;/sub&gt;</td>
</tr>
<tr>
<td>B-4-5A</td>
<td>Butte86ADA-11</td>
<td>B-4-5A</td>
<td>Butte86ADA-11</td>
<td>B-4-5A</td>
</tr>
<tr>
<td>Rhamnolipids</td>
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<td>&gt;500</td>
<td>31.3</td>
<td>7.8</td>
</tr>
<tr>
<td>SRE</td>
<td>31.3</td>
<td>7.8</td>
<td>31.3</td>
<td>7.8</td>
</tr>
<tr>
<td>SYRA</td>
<td>15.6</td>
<td>7.8</td>
<td>&gt;125</td>
<td>&gt;125</td>
</tr>
</tbody>
</table>

**Figure 4-3.** MIC determinations of SRE (I) and SYRA (II) with 0.2 % Tween<sup>®</sup> 20. The photographs were taken after 72 h incubation at 24 °C. Column 1: negative control (PDB + casamino acids + Tween<sup>®</sup> 20), column 2: positive control (PDB + casamino acids + Tween<sup>®</sup> 20, and 10<sup>4</sup> *F. graminearum* macrocinidia), and columns 3 to 12: 2-fold dilutions of SRE or SYRA (125-0.25 µg/ml in PDB + casamino acids + Tween<sup>®</sup> 20 and 10<sup>4</sup> *F. graminearum* macrocinidia). Rows A to D were tests against B-4-5A and E to H were against Butte86ADA-11. SYRA had no fungicidal activity, but controlled the pigment production of *F. graminearum*. 
Figure 4-4. Percent lesion areas developed in leaf infection assays with SRE and SYRA treatments. The lesion areas were measured by digital photography and analysis with APS Assess (plant disease quantification software, APS Press, St Paul, MN). Results of three separate experiments are shown. SRE and SYRA were applied to wheat seedlings at 5, 30, and 180 µg/ml (in 0.25 % [w/v] agar solution including 0.2 % [v/v] Tween® 20) with and without F. graminearum macroconidia. The results were variable in each experiment with different degree of phytotoxicities, but SYRA tended to prevent F. graminearum infection with relatively low phytotoxicity.
2nd experiment

![Graph showing percent lesion area for 2nd experiment.]

3rd experiment

![Graph showing percent lesion area for 3rd experiment.]

- **Cultivar**: Alsen, Frontana
- **Treatment**:
  - control
  - SRE-5
  - SRE-30
  - SRE-180
  - SYRA-5
  - SYRA-30
  - SYRA-180
  - control-F
  - SRE-5-F
  - SRE-30-F
  - SRE-180-F
  - SYRA-5-F
  - SYRA-30-F
  - SYRA-180-F

- Y-axis: Percent lesion area
- X-axis: Treatment
Figure 4-5. Relative chlorophyll contents in leaf infection assays with SRE and SYRA treatments. Results of three separate experiments are shown. The values were measured using a CCM-200 Chlorophyll Content Meter (OPTICSCIENCE, Tyngsboro, MA). SRE and SYRA were applied to wheat seedlings at 5, 30, and 180 μg/ml (in 0.25 % [w/v] agar solution including 0.2 % [v/v] Tween® 20) with and without F. graminearum. Despite their phytotoxicities, SRE and SYRA reduced the degree of disease in the 2nd and 3rd experiments.
2nd experiment

![Graph showing relative chlorophyll content for different treatments and cultivars in the 2nd experiment.]

3rd experiment

![Graph showing relative chlorophyll content for different treatments and cultivars in the 3rd experiment.]

Legend:
- Cultivar
- Alsen
- Frontana

Treatment:
- control
- SRE-5
- SRE-30
- SRE-180
- SYRA-5
- SYRA-30
- SYRA-180

Relative chlorophyll content
Figure 4-6. Growth from leaf segments with SRE treatments. Leaf segments treated with SRE were plated on PDA + casamino acids plates and incubated at 24 °C for 48 h. A: negative control (no inoculation), B: positive control (F. graminearum), C: 5 µg/ml, D: 5 µg/ml + F. graminearum, E: 30 µg/ml, F: 30 µg/ml + F. graminearum, G: 180 µg/ml, H: 180 µg/ml + F. graminearum. No fungal growth was observed from the leaf segments treated with SRE.
Figure 4-7. Growth from leaf segments with SYRA treatments. Leaf segments treated with SYRA were plated on PDA + casamino acids plates and incubated at 24 °C for 48 h. A: negative control (no inoculation), B: positive control (F. graminearum), C: 5 μg/ml, D: 5 μg/ml + F. graminearum, E: 30 μg/ml, F: 30 μg/ml + F. graminearum, G: 180 μg/ml, H: 180 μg/ml + F. graminearum. No fungal growth was observed from the leaf segments treated with SYRA.
CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

The main focus of this research was to test antifungal activities of newly synthesized aminoglycosides and syringomycin E (SRE) against *Fusarium graminearum* in head blight disease of wheat and barley. *In vitro* activities were estimated as minimum inhibitory concentrations (MICs) and *in vivo* activities were examined using Evans’ leaf infection protocol.

JL38 is a new aminoglycoside synthesized by glycodiversification of kanamycin B (3). It has an alkyl chain in ring III instead of the normal functional group of kanamycin B. JL38 showed better fungicidal activity against *F. graminearum* and lower phytotoxicity on host plants in *in vivo* assays than the substrate did. In the Takemoto lab, JL38 was tested against a broad spectrum of microorganisms and the inhibitory activities were mainly limited to fungi. This target specificity would avoid the emerging problem of resistance among environmental bacteria against aminoglycosides in general. The results suggest that JL38 is a strong candidate for control of *F. graminearum* in the field.

Three main future directions for research on JL38 are suggested. The first is determination of its mode of action against fungi. Such studies would include how it suppresses fungal growth and what factors make fungi susceptible to this drug. A second research direction is to develop methods that will improve the production of JL38. JL38 is currently synthesized by chemical methods and the yield is very limited for practical use. Finally, the third direction is testing the effectiveness of JL38 on different parts of the host plants. Spikes are the most important parts in wheat and barley.
cultivations as food productions, and they are also the most susceptible parts of the plants in anthesis (5). JL38 must be able to control infection on spikes to prevent economic loss by *F. graminearum*.

SRE showed better *in vitro* fungicidal activity against *F. graminearum* compared to JL38. *In vivo*, SRE prevented fungal infection but caused chlorosis on wheat leaves at all tested concentrations. SRE had been described as a phytotoxin, but recent studies indicate that SRE is instead an antifungal metabolite (2, 6). O’Donovan et al. (4) reported enhancement of retention and absorption of an herbicide with Tween® 20. Tween® 20, a wetting agent in the assay, possibly accumulates SRE in host plant cell and the resulting higher concentrations of SRE may lead to plant damages and phytotoxicities.

In MIC determinations, the antifungal activity of SRE was observed to increase 2-fold against one strain of *F. graminearum* in the presence of Tween® 20 as well as in the presence of rhamnolipid surfactants. On the other hand, SRE showed fungistatic but not fungicidal activity in the presences of both surfactants. The mechanisms how these surfactants interact with the activity of SRE are unknown. Since employment of surfactants in agrochemical applications is common, it is important to clarify their roles in the antifungal action of SRE.

Under treatment with SRE in rhamnolipid and Tween® 20 surfactant, *in vitro* pigmentation suppression and *in vivo* growth inhibition of *F. graminearum* were observed. Generally, pigments of this fungal growth are considered indications of mycotoxin production (1). According to the results, mycotoxins are possibly involved in the invasion of host plants by *F. graminearum*. The identification of the suppressed
mycotoxins will lead to a better understanding of *Fusarium* head blight disease.

References


5. **Parry, D. W., and P. J. L. McLEOD.** 1995. *Fusarium* ear blight (scab) in small grain cereals; a review. Plant Pathol. **44:**207-238.