Differential Pathogenicity of *Fusarium semitectum* in Texas Turtles

Thesis

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by

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I dedicate this manuscript to my mother, for it is her support that has given me the drive to continue my education. I would like to express my most sincere appreciation to my husband, Jason Smith, because he has given me much needed support and assistance in completing my research. I would also like to extend thanks to the rest of my family and my lab colleagues who have been a great network of support.

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ABSTRACT

The Texas tortoise, *Gopherus berlandieri*, is susceptible to a fungal infection that presents as a white lesion on the thin epidermal scutes. Box turtles residing in the same environment are not susceptible. The fungal infection can be transferred to human fingernails by handling infected tortoises. Morphological characterization and polymerase chain reaction (PCR) analyses of cultured fungal samples were used to determine and verify the identity of the fungus as *Fusarium semitectum.*

Pathogenicity of the isolated, cultured fungus was verified by applying samples of the cultured *F. semitectum* to shells of tortoises and box turtles. Growth occurred on the experimental sites of the tortoise shells but did not occur on the experimental sites of the box turtle shells. To determine if the fungal mycelium penetrated into the scutes, samples were taken from the experimental and control sites on the tortoise shell and viewed by scanning electron and confocal microscopy.

Experiments were performed to determine nutritional requirements of the fungus. Results indicated that the *F. semitectum* is able to consume tortoise shell and fingernail, but does not consume box turtle shell. Extraction and analyses of scute and nail keratins were performed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and no significant differences were found. Subsequent to demonstration that *F. semitectum* is a lipase producer, analysis of scute fatty acids was performed by reversed phase high-performance liquid chromatography (HPLC). Results showed significant differences in fatty acid components among box turtle scutes, fingernails and tortoise scutes. This suggests that one or more fatty acids found in box turtle scutes have anti-fungal properties.

INTRODUCTION

Evidence of shell disease caused by fungal organisms has been found in turtles and tortoises in various locations in the southern United States (Rosskopf, Jr., 1986). The Texas Tortoise, *Gopherus berlandieri*, is susceptible to a fungal infection (Rose *et al.*, 2000) that presents as a white lesion on the scutes --the thin epidermal plates covering the bony shell (Noble and Noble, 1940). The fungus infects both the carapace and the plastron, with the plastron becoming more heavily infected. Remarkably, box turtles residing in the same environment do not show signs of fungal infection. During the course of this study, it also became apparent that the fungus could be transferred from the tortoise to human fingernails by handling infected tortoises.

Some *Fusarium* species are common soil fungi known to cause dermal infections in both animals and humans. The genus *Fusarium* is characterized by the presence of hyaline septate hyphae, long or short phialides with a characteristic collarette visible at the apex, microconidia either in slimy heads or chains, and curved, multi-cellular macroconidia with a foot cell (St-Germain and Summerbell, 1996). The method of spore formation allows spores produced in slime to be dispersed by water and soil particles (Austwick, 1984). There are twenty-two *Fusarium* species that have been isolated and are known pathogens to animals; they commonly invade non-living or dying tissues. There are previous reports of *Fusarium* infections in turtles and tortoises. Rosskopf, Jr. (1986) reports cases in which *Fusarium* species have been isolated from a radiated tortoise and loggerhead turtles.

Dermatophytes are able to use keratin, a structural protein, as a substrate by invading and parasitizing keratinized tissue such as skin, nails and hair (Muhsin *et al.*, 1997). Previous studies have shown that the amino acid composition of reptilian keratin, including turtle scute, is very similar to mammalian keratin (Wyld and Brush, 1979; Gillespie *et al.*, 1982). The two main types of keratins are soft keratins and hard keratins. Reptilian scute and fingernail are both made up of hard keratins. Fungal dermatophytes typically produce

extra-cellular lipases in order to gain access and colonize keratinized tissue more efficiently (Muhsin *et al.* 1997; Das and Banerjee 1974). A study done by Grippa et al. (1999) tested the anti-lipase effects of selected natural substances on a microbial lipase, and found that some fatty acids have an inhibitory effect on lipase activity. Other previous research shows that fatty acids can inhibit respiration, pigment formation, fatty acid synthesis, carbohydrate metabolism, phosphate uptake, or neutral lipid metabolism in fatty acid sensitive fungi (Heseltine, 1952; Samson et al., 1955; Vicher *et al.*, 1959; Wirth and Anand, 1964; Vicher *et al.*, 1968). However, these effects suggest that there is a nonspecific interaction between the fatty acids and cellular enzymes because the effects of the fatty acids on the fungi had no apparent relationship to metabolic processes (Das and Banerjee, 1982).

These observations have lead me to the following hypothesis: since tortoises are susceptible and box turtles are resistant to infection of this particular fungus, then a component of the box turtle shell may be responsible for conferring the resistance to fungal infection. I tested this hypothesis by: 1) identifying the fungus using morphological characteristics and PCR; 2) performing *in vivo* and *in vitro* pathogenicity tests of the fungus on tortoise scute, box turtle scute and fingernail; 3) determining the nutrient requirements of the fungus; 4) extracting and analyzing proteins from host tissue; 5) performing a lipase assay to determine if the fungus is a lipase producer; and 6) extracting fatty acids and analyzing composition of fatty acids in host tissue. Results confirmed identity of the fungus as *Fusairum semitectum*. *In vivo* pathogenicity tests with this fungus showed the tortoise is susceptible and the box turtle is resistant. Pathogenicity tests performed *in vitro* showed that tortoise and fingernail are susceptible and box turtle is resistant to fungal infection. The fungus grew in a wide variety of environments and was not keratin-dependent. Protein extraction of tortoise scute, fingernail and box turtle scute showed no significant difference in the banding pattern of proteins present with relative molecular masses known for

keratins. Lipase assays showed *F. semitectum* is a lipase producer, and fatty acid extraction and analysis showed significant differences among box turtle scute, fingernail and tortoise scute.

METHODS

A. Identification of the Fungal Specimen

A sample of fungus from the keratin-supplemented agar plate was stained with Trypan Blue dissolved in lactophenol and viewed under a light microscope. Slides were made of the fungus cultured in the malt-extract culture medium and the Vogel's minimal medium (Vogel, 1956). Images from these slides were obtained by differential interference contrast (DIC) microscopy and recorded using NIH Image, a software package by NIH (http://rsh.info.nih.gov/nih-image/). Samples were sent to the Fungus Testing Laboratory in San Antonio, Texas, for morphological identification and to the United States Department of Agriculture in Peoria, Illinois, for PCR analysis.

B. Pathogenicity Tests

Three tortoises (*Gopherus berlandieri*) and seven box turtles (four *Terrapene carolina triungus* and three *Terrapene ornata*) were used to test the pathogenicity of the fungus in accordance with SWT Institutional Animal Care and Use Committee (approval # 99-02). The shells were prepared by cleaning them with a scrub brush and water. Three experimental sites and three control sites were randomly chosen on each tortoise shell, for a total of eighteen sample sites. Two experimental sites and two control sites were randomly chosen on each box turtle shell, for a total of twenty-eight sample sites. The sites were prepared by smoothing out the surface with a file and swabbing the area with ethanol. Wells were formed by cutting 1.5 ml polypropylene micro centrifuge tubes 1 cm from the top and gluing the portion with the attached closure to chosen sites on the tortoise and box turtle shells. The surface area of the bottom of the well was 2.01 cm^2. The wells were attached with epoxy, which was allowed to harden overnight. A spore suspension was made by adding deionized water to the keratin-supplemented agar plate and swirling. The spore concentration was 8×10^5 cells/ml, determined using absorbance at 600 nm (A_{600}) (Koke, 1971). The spore suspension was stored in a screw cap vial between uses. To each of the

wells chosen as an experimental site, 200 μl of the spore suspension was added. To each of the wells chosen as a control site, 200 μl of sterile deionized water was added. The tortoises and the box turtles were released into an outdoor habitat as shown in Figure 1. This habitat consisted of a shaded grassy area with sufficient cover and water. The subjects were observed at 14, 21 and 35 days post-inoculation. After 35 days post-inoculation, samples of tortoise scute were taken from the experimental site and the control site. The samples were mounted to a stud and sputter-coated with gold for 30 seconds prior to viewing with the scanning electron microscope (SEM). Images of varying magnifications were recorded.

Small samples of tortoise scute, box turtle scute and human fingernail were mounted on 3% agar plates (with no nutrient supplement). Control plates were not inoculated while experimental plates were inoculated with fungal mycelia. Approximately 7-10 days post-inoculation, the samples were removed from the agar plate and fixed in 4% paraformaldehyde. The samples were rinsed thoroughly in phosphate buffered saline (PBS) and stored in 30% sucrose in preparation for cryosectioning. After the samples were sectioned, they were mounted on gelatin-coated cover slips and stained with 2 mg/ml Fluorescent Brightener #28 (Sigma, St. Louis, Missouri) in ethanol. The cover slips were allowed to dry for one hour and were rinsed in 70% ethanol. The cover slips were mounted on glass microscope slides and viewed using epifluorescence.

Figure 1

Images of the study sites used during the pathogenicity study. The first image is the site where all three tortoises and one box turtle resided (A). The second image is the site where six box turtles resided (B). The subjects were observed over a total of seven weeks in order to determine the presence or absence of *Fusarium semitectum.*

A

B

C. Nutrient Requirements

A sample of fungus was taken from the scute of an infected tortoise and plated on a keratin-supplemented agar plate. Another sample was plated on a malt-supplemented agar plate. Malt-extract culture media was made by thoroughly mixing 15 g/l dehydrated malt extract broth with deionized water. After the media was autoclaved, dextrose was added. A sample of fungus (taken from a colony grown on the keratin-supplemented agar plate) was placed in the culture media. Stock salt solution was mixed with deionized water to make Vogel's minimal media. The media was then autoclaved. Dextrose was added to the culture media. A spore suspension was made by adding Vogel's minimal medium to the keratin-supplemented agar plate and swirled. The spore concentration was 4×10^5 cells/ml. The spore suspension was then placed in the media. Both malt-extract and Vogel's minimal media culture flasks were placed on an agitator for five days, at which time samples were observed microscopically, using DIC, for presence of fungal mycelia.

D. Protein Extraction and Analysis of Host Tissue

Keratin extraction and protein analyses were performed on tortoise scute, box turtle scute, and fingernail by SDS-PAGE. The samples were prepared by washing in 10% Triton X 100 and rinsed thoroughly with water, ethanol and petroleum ether. Dried samples were weighed and approximately 60-70 mg of washed keratin was extracted using 5 ml of mercaptoethanol reducing solution at pH 10.5, as described by Thorpe and Giddings (1983), for 12 hours at 37°C using a shaking water bath.

The extracts were subjected to electrophoresis as described by Bolanos et al., (1998), with the following modifications. The samples were mixed with sample buffer, boiled for 10 minutes and briefly centrifuged to remove solid debris. The samples (10 μl aliquots) were then applied to two SDS polyacrylamide gels (12% separating, 3% stacking). The proteins were separated by applying 200 V for approximately 45 minutes, or until the bromphenol blue dye front reached the bottom of the gel. The gels were placed in

Coomassie blue stain for two hours and de-stained in 10% methanol and 10% acetic acid.

E. Lipase Analysis of *Fusarium semitectum*

Slant agar tubes (3%) were prepared by mixing agar (no nutrient supplement) and Vogel's minimal media. Olive oil was added to the experimental tubes in order to induce production of lipase if the fungus is a lipase producer. The tubes were inoculated with *F. semitectum.* The culture was allowed to grow for 10 days before samples were taken. A sample was taken by swirling 2 ml deionized water in the tube and pouring into 1.0 ml aliquots to be used in the lipase assay. Lipase substrate and Trizma buffer were purchased from Sigma (St. Louis, Missouri). The assay was done by pipetting 2.5 ml water, 3.0 ml lipase substrate, and 1.0 ml Trizma buffer into each of two test tubes, labeled "experimental" and "blank". A 1.0 ml aliquot of the fungal spore suspension was added to the experimental tube. Both tubes were capped and mixed by shaking vigorously. They were placed into a 37°C waterbath for 6 hours. Then a 1.0 ml aliquot of the fungal suspension was poured into a flask, labeled "blank", and refrigerated. After the six hour incubation period, the contents of the "blank" tube were poured into the "blank" flask, and the contents of the "experimental" tube were poured into a clean flask. Next, 3.0 ml 95% ethanol were added to the tubes, shaken to rinse, and poured into their respective flasks. Four drops of thymolphthalein indicator solution were added to each flask. The samples were titrated with 0.05 N sodium hydroxide until they turned a light blue. Both samples were titrated to the same color intensity. The volume of sodium hydroxide necessary to change the color was recorded. The lipase activity was calculated by taking the difference between the volumes of sodium hydroxide (experimental - control) per unit time. The lipase activity is defined as the amount of hydrogen ions produced per unit time. Two controls (an elevated lipase level and normal lipase level, purchased from Sigma, St Louis, Missouri) were also tested.

F. Fatty Acid Extraction and Analysis of Host Tissue

Fatty acids were extracted from the host tissue by washing pieces of tortoise scute, box turtle scute and fingernail in ethanol three times. The washes were collected in glass vials which were placed in a vented hood to evaporate the ethanol to dryness. Methanol:water (50/50) was added to the vials in order to redissolve the fatty acids. Aliquots of 50 μl were analyzed by HPLC using a C-8 reverse-phase column (5 cm x 4.6 mm) with a 100 μl loop. The column was equilibrated at a flow rate of 0.8 ml/min. The mobile phase consisted of 50/50 methanol in water with 0.1% trifluoroacetic acid. The eluate was monitored at the wavelength of 215 nm.

The instruments used to perform the HPLC analyses were a Gilson 7125 injector equipped with a Gilson 118 UV/VIS detector and a Gilson 306 pump. Data was recorded and analyzed using Gilson Unipoint software program. All analyses were done at room temperature.

RESULTS

A. Identification of the Fungal Specimen

The fungus was identified as *Fusarium* by microscopically examining morphological characteristics, described by St-Germain and Summerbell (1996), of the fungus cultured from the infected tortoise. Dr. Michael Rinaldi and the Fusarium Research Center confirmed the identification morphologically as *Fusarium semitectum.* Elizabeth Cigelnik at the Natural Center for Agricultural Utilization Research (NCAUR) in Peoria, IL, verified the identity by using polymerase chain reaction (PCR) of ribosomal DNA polymorphisms. Images obtained using DIC and epifluorescence revealed the presence of hyaline septate hyphae, a phialide with a collarette visible at the apex, and curved macroconidia were observed (Figure 2).

B. Pathogenicity Tests

All of the control sites of the pathogenicity tests, on both tortoise and box turtle hosts, showed no evidence of *F. semitectum* mycelia. All fourteen of the experimental sites on the box turtles showed no evidence of *F. semitectum* infection (Figure 3). Seven of the nine experimental sites on the tortoises resulted in the presence of *F. semitectum* (Figure 4). Scanning electron micrographs showed the absence of *F. semitectum* on the control sites (Figure 5) and the presence of *F. semitectum* mycelia on the experimental sites of the tortoise scute (Figure 6). Epifluorescent and confocal images also indicated the presence of *F. semitectum* in the tortoise shell and fingernail and the absence of *F. semitectum* in the box turtle shell (Figure 7).

Figure 2

Image A is from a section of the slide prepared from the *Fusarium semitectum* grown in malt extract culture medium. It shows a phialide with a collarette visible at the apex (yellow arrow). Image B is from a section of the slide prepared from the *F. semitectum* grown in Vogel's minimal medium. It shows the hyaline septate hyphae (red arrows). The images were collected by DIC and recorded using NIH Image. Image C, from a section of infected tortoise shell, shows a curved macroconidia. This image was collected using epifluorescence.

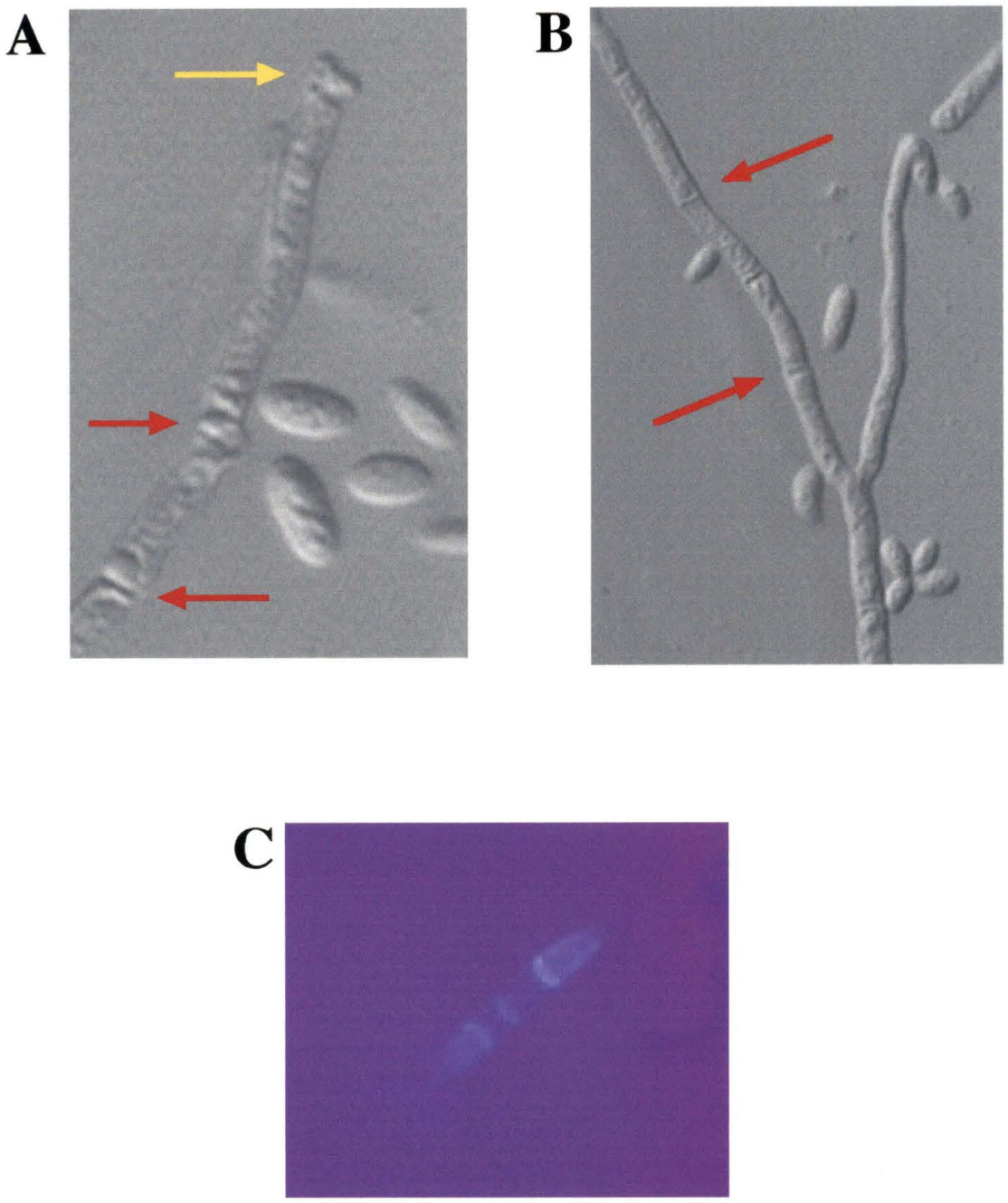
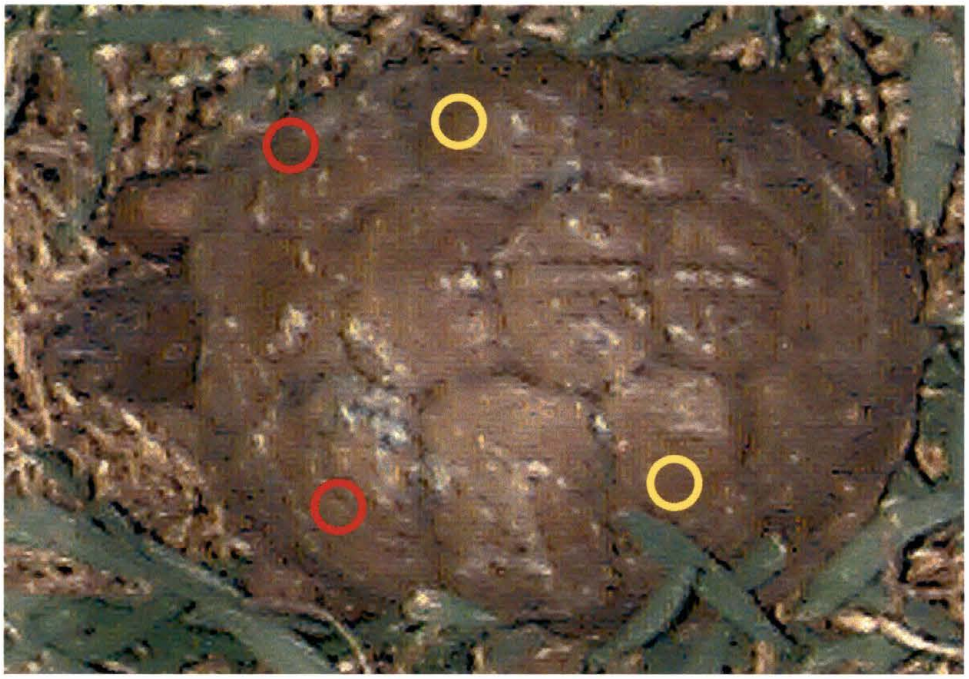

A
B
C

Figure 3

Image A shows the experimental design set-up for the *in vivo* portion of the pathogenicity study of the box turtle. Image B shows a box turtle with no evidence of the presence of *Fusarium semitectum* on the experimental sites (yellow) and control sites (red). The white seen on the shell is a residue left from the adhesive tape that was used to keep the tops of the wells closed.

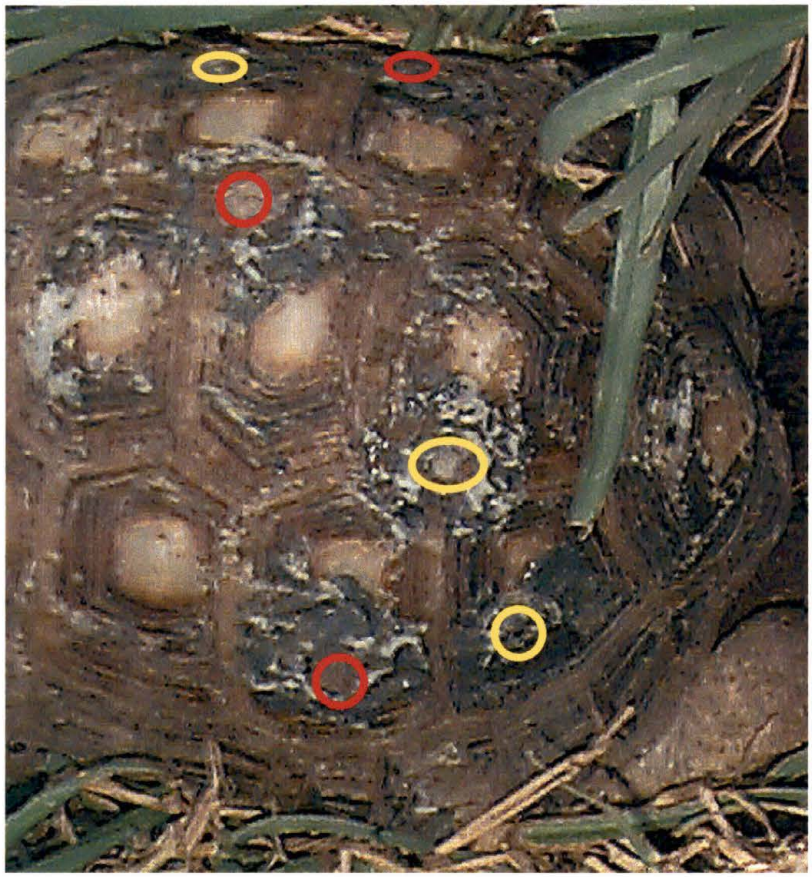


B

A

Figure 4

Image A shows the experimental design set-up for the *in vivo* portion of the pathogenicity study of the tortoise. Image B shows a tortoise with evidence of *Fusarium semitectum* on the experimental sites (yellow) and no evidence of *F. semitectum* on the control sites (red). The white seen on the shell is a residue left from the adhesive tape that was used to keep the tops of the wells closed.



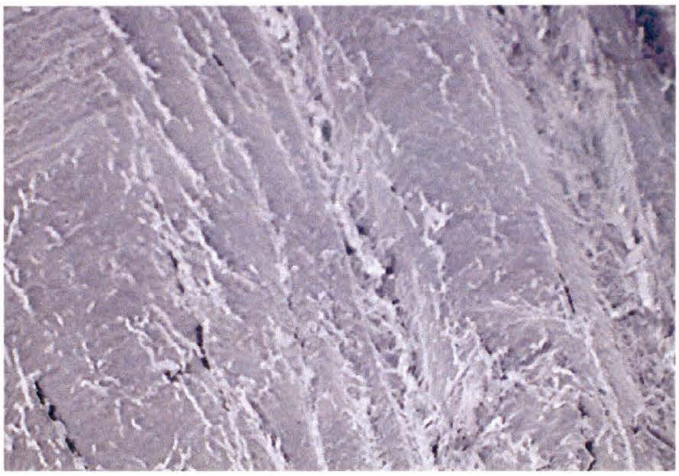
B

A

Figure 5

Scanning electron micrographs showing the absence of fungus on the control sites of the tortoise shell. Micrograph (A) is shown at the original magnification of 105X and (B) at original magnifications of 50X and 200X.

A



B

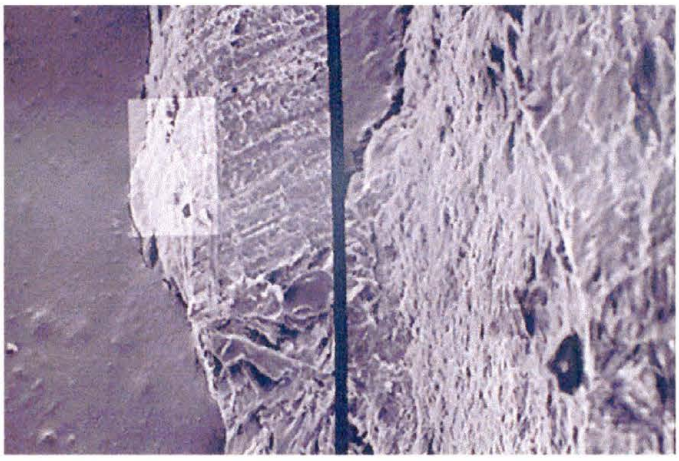
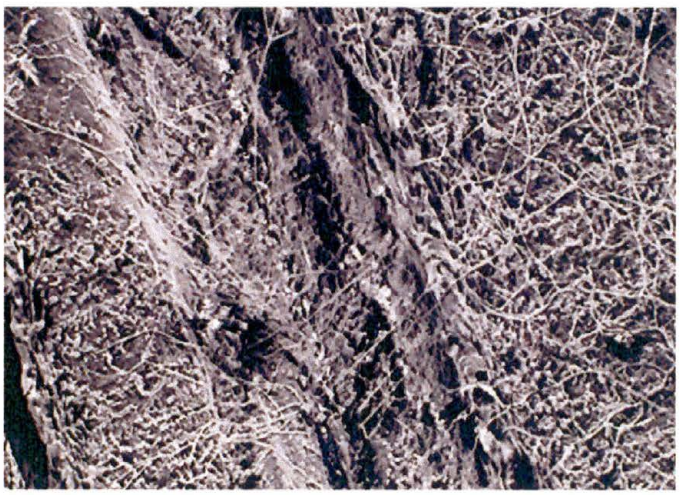


Figure 6

Scanning electron micrographs showing the presence of fungus on the experimental sites of the tortoise shell. Micrograph (A) is shown at the original magnification of 100X and in (B) at original magnifications of 200X and 1600X.

A



B

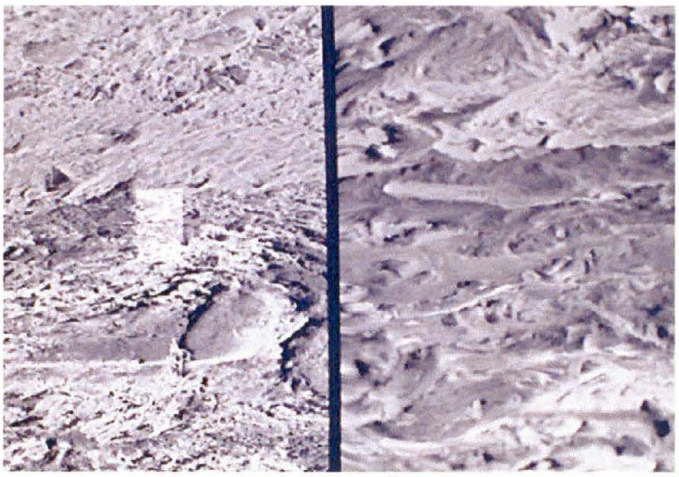


Figure 7

Epifluorescent images taken from a section of (A) tortoise shell, (B) fingernail and (C) box turtle shell. The images of the tortoise shell and the fingernail show the presence of *Fusarium semitectum*, whereas there was no evidence of the *F. semitectum* on the box turtle shell.

A

B

C

C. Nutrient Requirements

The presence of *F. semitectum* mycelia was observed in all of the cultures with the exception of the malt-supplemented agar plate. However, the malt-supplemented agar plate did show the presence of other fungal specimens, leading to the assumption that the culture may have been contaminated. Thus I concluded that the fungus is not keratin dependent, but able to metabolize keratin if available.

D. Protein Extraction and Analysis of Host Tissue

Keratin extraction and protein analysis, using SDS-PAGE, resulted in at least three comparable bands with molecular masses (M_r) of 70,000, 65,000, and 15,000 in all three of the host lanes (Figure 8). The molecular masses of the keratin-like components were estimated by comparing their mobility with that of the standards used.

E. Lipase Analysis of *Fusarium semitectum*

The results of the lipase assay show that *F. semitectum* is able to secrete lipase into its environment. The lipase activity determined under the conditions of the assay indicated *F. semitectum* has a lipase activity of approximately 2.1 units/hour (see "Methods" for the definition of lipase activity).

F. Fatty Acid Extraction and Analysis of Host Tissue

The HPLC analyses indicated two distinct populations of fatty acids in the box turtle sample (at 1.5 and 2.25 minutes), but only a single population in tortoise and fingernail samples (see Figure 9).

Figure 8

SDS-PAGE of extracted keratin from fingernail, box turtle shell and tortoise shell.

The molecular masses of the keratin-like components were estimated by comparing their mobility with that of the standards used.

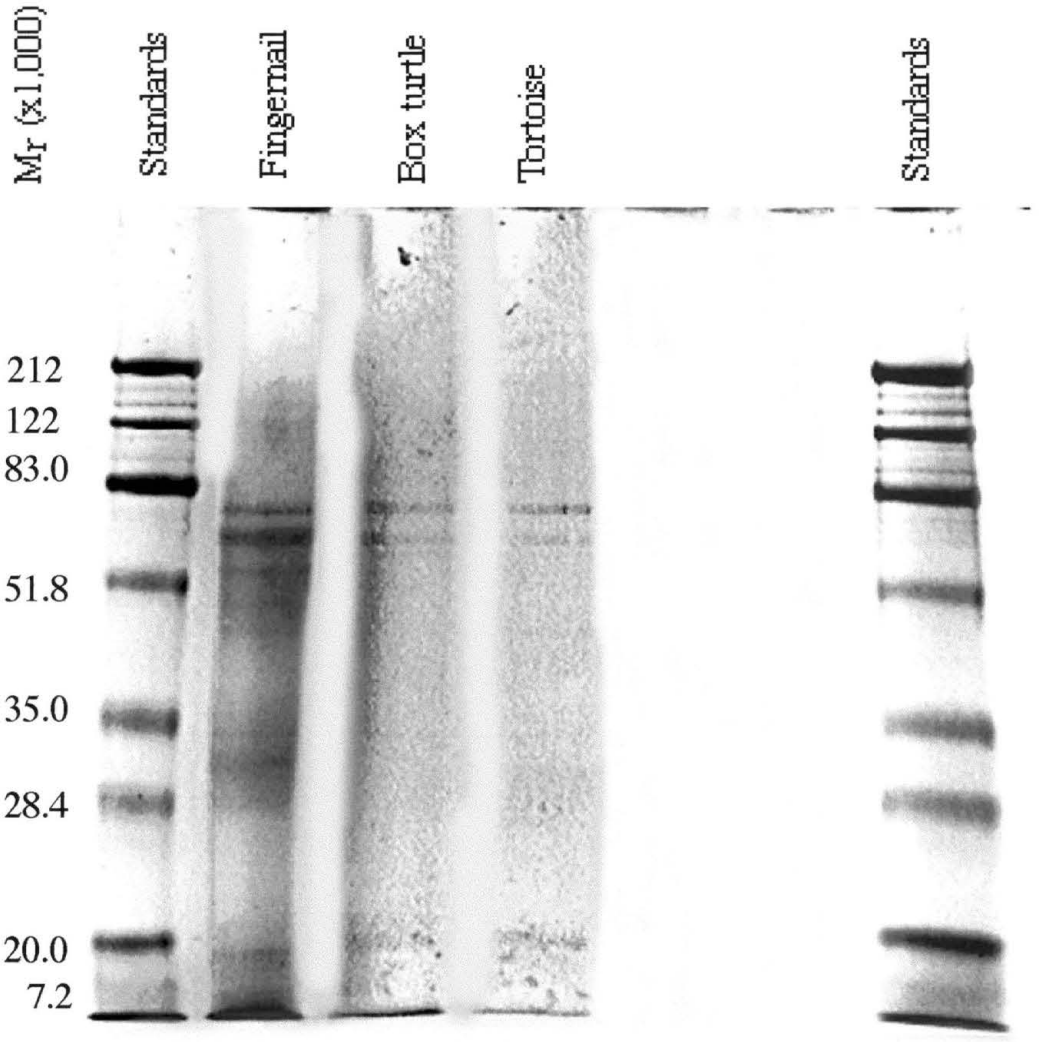
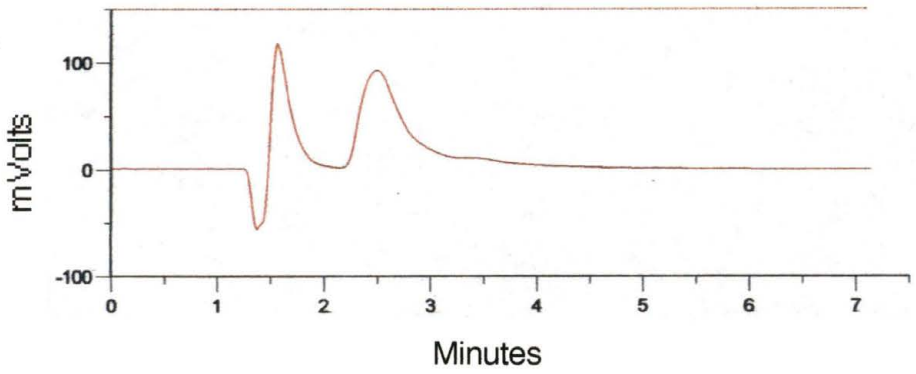

Mr (x1,000)
Standards
Fingernail
Box turtle
Tortoise
Standards
212
122
83.0
51.8
35.0
28.4
20.0
7.2

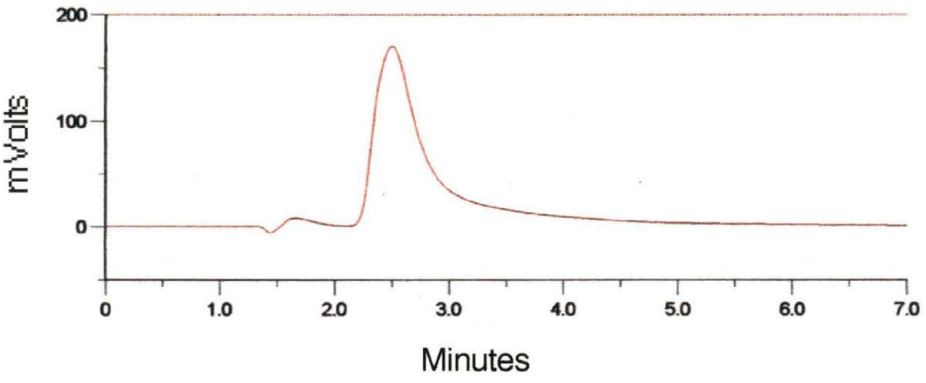
Figure 9

HPLC chromatograms of fatty acids extracted from box turtle (A), tortoise (B), and fingernail (C). The chromatogram of the box turtle sample shows two peaks. The chromatograms of the tortoise and fingernail samples show one peak each.

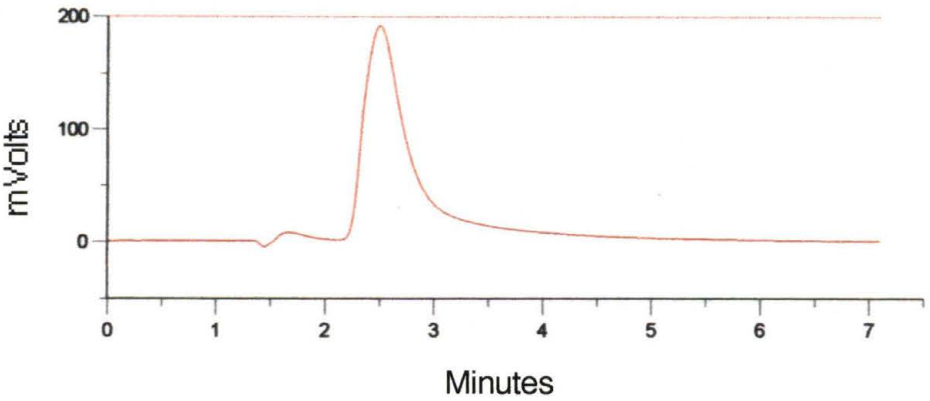
A


100
0
-100
mVolts
0
1
2
3
4
5
6
7
Minutes

B


200
100
0
mVolts
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Minutes

C


200
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mVolts
0
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2
3
4
5
6
7
Minutes

DISCUSSION

Morphological features and PCR analysis support the identification of the fungus as *Fusarium semitectum.* The fungus grew in all of the environments that it was introduced to with the exception of the malt-supplemented agar plate. The results of the pathogenicity tests and scanning electron microscopy indicate that tortoises are susceptible to *F. semitectum* infection while box turtles are resistant. The epifluorescent images verified that *F. semitectum* penetrates tortoise scute and fingernail, whereas it does not penetrate box turtle scute. SDS-PAGE analyses of extracted keratin from the hosts show no significant differences in the type of keratin-like proteins present in tortoise scute, box turtle scute and fingernail. The results of the HPLC analyses of fatty acids showed the presence of two peaks in the box turtle shell, whereas only one peak in both tortoise scute and fingernail.

Previous studies have shown several known types of *Fusarium* infections of reptiles, most commonly turtles, including eye and skin infections (Austwick, 1984). Rosskopf, Jr. (1986) reports a case in which Florida Gopher tortoises (*Gopherus polyphemus*), related to *G. berlandieri*, have been diagnosed with fungal shell disease.

Analyses of keratins and fatty acids from turtle scutes and reptile skin have also indicated similar results as in this study. For example, Thorpe and Giddings (1981) showed molecular weights of S-carboxymethylated keratin from shed reptilian skin have bands in one group at 11-20 kD and another group at 32-69 kD. Das and Banerjee (1983), showed that some long chain fatty acids have an anti-fungal property and are often used for the treatment of fungal infections.

The results from my research can extend current findings because they show: 1) Texas tortoises are susceptible to *F. semitectum*; 2) keratin is not a metabolic requirement for the survival of *F. semitectum*; 3) differences in keratin-like protein components of the three hosts are not responsible for the facilitation or inhibition of *F. semitectum* growth; and

4) a difference exists in the fatty acid composition of tortoise scute and box turtle scute, and this difference may be responsible for inhibition of *F. semitectum* in box turtles.

These findings are significant because they provide a better understanding of an important pathogen that affects tortoises in south Texas, and a better understanding at the molecular level of susceptibility to fungal infection. There is potential practical importance of this understanding in designing an anti-fungal treatment.

The results of this work support my hypothesis. A component of box turtle scute has been found that prevents infection by *F. semitectum.* Further experiments would include identification of the component, or components, that make up the unique fatty acid population found in the box turtle scute.

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