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Natural and Synthetic Viniferins Associated with the Grapevine Disease Young Vine Decline

David Michael McGinnis
Old Dominion University

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NATURAL AND SYNTHETIC VINIFERINS ASSOCIATED WITH THE GRAPESVINE DISEASE YOUNG VINE DECLINE

by

David Michael McGinnis
A.S. August 2000, Tidewater Community College
B.S. May 2002, Old Dominion University

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Old Dominion University in Partial Fulfillment of the
Requirement for the Degree of

MASTER OF SCIENCE

CHEMISTRY

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December 2005

Approved by:

__________________________
Roy L. Williams (Director)

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Mark S. Elliott (Member)

__________________________
James H. Yuan (Member)
ABSTRACT

NATURAL AND SYNTHETIC VINIFERINS ASSOCIATED WITH THE GRAPEVINE DISEASE YOUNG VINE DECLINE

David Michael McGinnis
Old Dominion University, 2002
Director: Dr. Roy L. Williams

Grapevine disease has been the subject of intense research amongst viticulturists over the last few decades, especially during the 1990’s. There has been discoveries that suggest grapevine disease is commonly caused by fungal pathogens. One of the most common fungi that the vine may become infected by is known as Botrytis cinerea. B. cinerea is capable of attacking the grapevine which in turn will lead to bunch rot in the grape clusters. This disease has been researched in great detail during the past several years and is one of only few microorganisms that have actually been identified.

Phaeoacremonium chalmydosporum is another fungal pathogen that affects grapevines. This fungal pathogen is the culprit that leads to the young vine decline disease in grapevines. The microorganism has the ability of infiltrating through a pruning wound on the trunk of the vine and then subsequently interacts with vine phytoalexins as the vine tries to counteract the invading pathogen. As a result, the xylem exudes a darkly colored viscous substance that is known as Black Goo. Black Goo is an obtrusive substance that inhibits the vine’s vasculature so that nutrient flow is reduced. This thesis will shed light on the chemical composition of the Black Goo exudate and the relationship with the fungal mechanisms in the plant. This research will present evidence
that *P. chalmydosporum* may have the ability to produce *trans*-resveratrol and certain natural viniferins which may be associated with the principle of self-intoxication and the progress of the fungal infection.
“An investment in knowledge always pays the best interest.”
-Benjamin Franklin

“Beer is evidence that God loves us and wants us to be happy.”
-Benjamin Franklin
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Writing a thesis is quite a daunting task and it will stand for the research that the writer has undertaken during their graduate career. Fortunately, I have been surrounded by great people during the past few years while at Old Dominion and those folks have made my graduate experience enjoyable. I will take the next couple of pages to acknowledge them.

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as I embark on my doctorate. I will do the best to make you proud. You will always be considered as my mentor and friend.

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INTRODUCTION

BACKGROUND ON BOTRYTIS CINEREA

Wine has been consumed dating back to ancient times. This delightful beverage has been known to help people relax after a long day of work and has also helped in maintaining sanity with some folks. Wine may also have positive ramifications in the health of those that drink it in moderation (1). This drink has been rationalized by many to be healthy enough to consume due to the concentration of anti-oxidants within every glass.

The wine industry has been experiencing problems in crop yield during the modern era which may have a tremendous impact on vineyard owners. Grapevines have been experiencing its fair share of interactions with pests and the most commonly associated pest of the grapevine is that of pathogenic fungi. Botrytis cinerea is a fungal organism that has wreaked havoc in plants worldwide and it is the most commonly studied do to its diversity in the hosts it affects (1).

Botrytis cinerea is a well known organism of the Botrytis genus and it is known to be pathogenic to grapevines. B. cinerea fungi may grow on dead leaf tissue of the vine canopy allowing for their conidia to be released into the air, which may then accumulate on the fruit during the growing season. According to Goetz et al., grape clusters can be infected and destroyed by B. cinerea; however, the infection is dependent on climatic conditions and the sensitivity of the grape variety (2). This fungal disease is commonly referred to as grey mold (Figure 1) and it has been known to infect tomato, kiwi, and

The Journal of Agricultural and Food Chemistry was used as the model for this thesis.
Figure 1. Photograph of grapevine infected with *Botrytis cinerea.* (Photo by J.LaMar). The arrow indicates the site of infection.
other plants as well. *B. cinerea* infection in certain white grape varietals helps to produce a sweet wine which has been important in the development of various dessert style wines. *B. cinerea* effects grapevines during the humid months of the year and will colonize rapidly once it settles on immature grapes. These grape bunches will become covered with the grey mold and will eventually darken and wilt as a result. Thus, grape yields are greatly diminished and the resulting wine would taste moldy. Prized *Botrytized* wines are referred to as being produced by the noble rot. Noble rot will occur during a short period of rain or humidity in the mid to late season where grapes are more ripe than green. This period will be followed by a sustained cool and dry period where the average temperature hovers around 60°F. Under the conditions, the *B. cinerea* penetrates the grape skins with mycelia in order to feed and scavenge water from the grape which consequently causes the grape to shrivel. The acidity of the grape will plummet and the formation of gums and citric acids will lead to an enhanced concentration of grape sugar. Thus, the increased sweetness will inhibit the speed of the fermentation process where high concentrations of glycerol are obtained over an extended period of time and will make a fine dessert wine as a result.

**BACKGROUND ON YOUNG VINE DECLINE**

Young vine decline (a.k.a. Black Goo) was first identified by Leonardo Petri in 1912. Petri stated that the wound related gummosis and wood streaking are signs of this grapevine disease (3). Petri discontinued this research shortly thereafter and further research pertaining to young vine decline was not revived until Dr. Laura Mugani published a paper on Petri’s disease in 1998 (3). Young vine decline is a destructive grapevine disease of the woody tissue and has been reported to cause serious loss of
grapevines worldwide in vines ten years old or less (4). This disease has started to become a major problem amongst vineyard owners due to its ability of reducing crop yields and its role of causing the premature death of grapevines.

Young vine decline is known to be caused by the fungus, *Phaeocreamonium Chlamydosporum*, that infects grapevines which are less than ten years old (4). This fungal pathogen has the ability of invading the grapevine trunk through pruning wounds which gives it the ability to rapidly attack the xylem. The fungal infection leads to the brown streaking of the trunk which Petri observed in 1912 (3). A gummosis or tylose material is produced internally as a result of the fungal infection and is exuded into the xylem over a period of time (5). Tyloses lead to the obstruction in the vessels of the grapevine and subsequently leads to the formation of a black viscous material in the xylem (Figure 2). This obstruction causes transport problems with regards to water and nutrients in the vascular system and will thus lead to the premature death of the vine (5).

*P. chlamydosporum* (Figure 3) is a potential airborne fungus. According to Pascoe, the fungus is able to penetrate into the grapevine via pruning wounds during the winter and contaminations seem to be correlated with rainfall (6). This mode of entry therefore makes it possible for there to be rapid spreading of the fungi throughout the vineyard. The *Phaeocreamonium* genus is relatively new and it was first described in 1996 by Crous et al. (7). None of the *Phaeocreamonium* species are known to have a sexual state which makes them teleomorphic. According to Pascoe, this genus contains colored pigmented phialides, colorless spores, and a simple life cycle (Figure 4) (6).

Fungal conidia infiltrate the host tissue and xylem vessels which lead to the production of tyloses and phenolics by the host. These tyloses and phenolics lead to production of the
Figure 2. This is a sample grapevine infected with young vine decline. The arrow indicates the site of infection.
Figure 3. *P. chlamydosporum* cultured onto Potato dextrose agarose plates.
Figure 4. *P. chlamydosporum* lifecycle.
black gummosis that was discussed earlier. Conidiophores and conidia (Figure 5) will be produced on the exposed grapevine surfaces that are pruned, which would then provide an opportunity for the fungal conidia to be displaced by rain. This displacement will increase their ability to travel to other unaffected grapevines (6). Young vine decline poses as a great threat to the wine industry since there has not been a great deal of research on the topic.

**BACKGROUND ON PHYTOALEXINS (TR)**

Phytoalexins are low molecular weight antifungal secondary metabolites that have been shown to possess biological activity against a wide range of pathogens and can be considered as markers for plant disease resistance (8). 3,4′,5-trihydroxy-trans-stilbene or trans-resveratrol (TR) is a commonly known phytoalexin found in grapevines. Daniel et al. reported that TR levels may reach concentrations of 50 to 400 μg/g fresh weight of infected leaves (9). TR is a compound that is associated with the so called “French Paradox” as related to the moderate consumption of red wine. French citizens have a tendency to eat foods that are high in cholesterol which may eventually lead to chronic heart disease over a period of time. It was reported by Siemann and Creasy that TR is a constituent of Chinese oriental folk medicines and was used to treat people that were inflicted with a range of disorders such as those affecting the liver, skin, heart, circulation, and lipid metabolism (10-12). Goldberg et al. stated that moderate red wine consumption could lead to a significantly lower risk of incidence of coronary heart disease (CHD). According to epidemiologic evidence that this group collected, the presence of these unique polyphenolics was the basis for the so called French paradox (13).
Figure 5. *P. chlamydosporum* conidia under light microscopy.
TR naturally occurs within grapevines that become infected or stressed in some specific fashion. For instance, TR is synthesized by the vine as a result of UV irradiation or microbial infection. Grapevines have the ability of synthesizing carbohydrates, lipids, and proteins and are known to be capable of producing phenolic compounds from certain lipid and aromatic precursors. These grapevine phenolics appear to protect the vine against UV light and exhibit biocidal effects against bacteria and fungi (9). According to plant physiologists, the production of TR in stressed grapevines follows the Shikimate pathway (14,15,31). In this pathway (Figure 6), phenylalanine is the starting point for the TR synthesis which is first deaminated to give cinnamic acid via a phenylalanine ammonia lyase. Cinnamic acid is then hydroxylated at the para carbon to form coumaric acid and three parts malonyl-CoA. The final and most important step in TR synthesis is dependent on a stilbene synthase enzyme. This pathway has given researchers a basis for understanding the role of TR in the development of the disease resistance in grapevines. It appears that TR is the major player in the development of a disease resistance mechanism in grapevines, however, other phytoalexins which are not derived from the Shikimate pathway may also be involved in their disease resistance.

BACKGROUND ON NATURAL VINIFERINS IN GRAPEVINES

Langcake and Pryce are considered by many to be pioneers in the field of grapevine physiology. They have published several papers between 1977 and 1981 that have been important to the field of plant research. Langcake and Pryce discovered a new set of phytoalexins in the *Vitis vinifera* vine which were classified as viniferins (16). Extraction of grapevine leaves infected with *B. cinerea* led them to conduct a bioassay of the extract using silica gel thin layer chromatography. The group established that one of
Phenylalanine ammonia lyase

Cinnamate-4-hydroxylase

CoA SH

CoA Ligase

Stilbene synthase

3HOOC-CH₂CO-SCOA

trans-resveratrol

Figure 6. Shikimate pathway for the production of stilbenes (i.e. TR).
the major components of the extract was identified as ε-viniferin (1,3-benzenediol, 5-[(2R,3R)-2,3-dihydro-6-hydroxy-2-(4-hydroxyphenyl)-4-[(1E)-2-(4-hydroxyphenyl)ethenyl]-3-benzofuranyl)] (Figure 7) and was isolated as a homogeneous amorphous solid with a melting point of 155-160 °C (16). ε-Viniferin has a molecular formula of C_{28}H_{22}O_{6} and has a parent ion of 454 m/z (22). This compound is considered to be a TR dehydrodimer that is formed through resorcinol oxidative coupling of TR in the plant. There is one other major oligomeric compound that has been characterized from the diseased grapevine leaves. α viniferin (C_{42}H_{30}O_{9}; cyclononal[1,2,3-cd:4,5,6-c'd':7,8,9c''d''] trisbenzofuran-4,9,14-triol,2,2a,7,7a,12,12a-hexahydro-2,7,12-tris(4-hydroxyphenyl)(2R,2aR,7R,7aR,12S,12aS)-rel-(+) (Figure 7) was the other major phytoalexin found in grapevines infected with B. cinerea (17). Neither TR nor the ε and α viniferins could be detected by these researchers during their study of healthy grapevines. This would suggest that these viniferins are produced as a means for the development of disease resistance (18).

Grapevine resistance has been studied intensively during the last few decades, especially with regards to various sources of fungal infection. Current research published in numerous journals has suggested that the accumulation of viniferins take place once the vine becomes infected. It has been stated by Jeandet et al. that phytoalexins possess a great deal of biological activity against a variety of pathogenic organisms and are considered to be a marker for disease resistance (19). Langcake and Pryce reported that the effective dose to be required for 50% mortality of TR upon dormant conidia of B. cinerea was greater than 200μg/ml (20). The authors further mentioned that TR is a precursor for viniferin production. Thus, it seems that the grapevine produces viniferin
Figure 7. Phytoalexins that have been detected in stressed grapevines associated with *B. cinerea.*
compounds as a way of defending itself against fungal pathogens. In 1981, Langcake established that ε-viniferin has an antifungal activity upon germination of *B. cinerea* conidia of 36 μg/ml (21). This mortality rate drops significantly with the inoculation of ε-viniferin into *B. cinerea* and not with the inoculation of TR. There may well be other viniferins that are produced as a result of fungal infection that have not been identified to date.

Pezet et al. discovered yet another viniferin that they classified as δ-viniferin (1,3-Benzenediol,5-[(1E)-2-[(2R,3R)-3-(3,5-dihydroxyphenyl)-2,3-dihydro-2-(4 hydroxyphenyl)-5-benzofuranyl]ethenyl] (Figure 7) from grapevines infected with *Plasmopara viticola*, which is otherwise known as downy mildew. This group reported that this viniferin was almost as significant in the plant extract when compared to that of ε-viniferin and is considered one of the most important phytoalexins produced from resveratrol (22). These researchers report that δ-viniferin is produced in vitro by the oxidative dimerization of TR by plant peroxidases or fungal laccases (22). δ and ε viniferin are isomeric, thus, both viniferin compounds share the same parent ion in mass spectrometric analysis. Pezet et al. was able to enzymatically synthesize δ-viniferin from TR using a peroxidase, which is similar to the natural enzyme found in plants (22). δ-viniferin is another type of TR-E-dehydrodimer that is produced through phenolic coupling during the oxidative dimerization. This viniferin was reported to have a concentration of 155.68 mg/g in *P. viticola* infected grapevine leaves (22). There will be a comment on the mechanism(s) for viniferin synthesis in the discussion portion of this thesis due to their importance in disease resistance.
RESEARCH OBJECTIVES

This thesis was developed based on our interest in young vine decline disease in Virginia grapevines. There has been a copious amount of research carried out in grapevines infected with *B. cinerea*, however, research in the case of young vine decline is still somewhat limited. Our first objective was to obtain samples of grapevines that were infected with young vine decline. The current literature has been mostly geared toward the extraction of leaves that are infected with *B. cinerea* and the determination of the amount of the various phytoalexins present in these leaves. However, the nature of the Black Goo material that exudes through the xylem in vines infected with young vine decline has not been fully characterized. Our second objective is to isolate and analyze the chemical constituents associated with young vine decline via high pressure liquid chromatography (HPLC) of the Black Goo exudate. HPLC is a highly effective analytical tool that will enable us to determine the complex nature of the exudate as to the retention time and polarity of the various fractions. This information may allow us to explain or propose a mechanism to account for the formation of these compounds in the infected grapevine. Our third objective is to further characterize each of the Black Goo fractions using liquid chromatography/mass spectrometry (LC/MS). This will provide us with the parent ion of each fraction and will help in the determination of its chemical structure. The fourth objective was to carry out a similar chemical analysis of *P. chlamydosporum*. This fungal pathogen has been isolated from the infected grapevine aseptically. Samples of the pathogen will be sonicated, extracted and analyzed using HPLC in order to ascertain the chemical components of the fungal organism.
MATERIALS AND METHODS

PLANT MATERIAL

Syrah grapevines (number 3309) were collected during spring 2003 from Horton vineyards located in Orange, Virginia. These grapevines were between four and five years of age and were showing signs of young vine decline. The grapevines were removed and cut into sections to confirm that they were in fact infected with Black Goo.

FUNGAL ISOLATION AND IDENTIFICATION

*Phaeoacremonium chlamydosporum* was aseptically isolated from the exterior woody portion of the infected Syrah grapevine section. *P. chlamydosporum* was inoculated onto potato dextrose agar plates (BD Difco Potato Dextrose Agar; 39g suspended in 1L water and autoclaved for 15 minutes at 121 C. The pH of the suspension was 5.6 ± 2. Each of the inoculated plates was incubated at 35 C. Mugani has reported that the fungus will form a white shiny, yet somewhat elevated surface during the initial stages of growth which will then progressively turn to a dark olive green color as the fungus matures. This process usually takes a week (23). The isolated fungus from the Syrah vines exhibited these same morphological characteristics (i.e. color, shape, etc.) as described in Mugani’s research.

HPLC INSTRUMENTATION

Analysis of the crude young vine decline exudate was carried out by HPLC using a Beckman Gold unit fitted with a C18 reversed phase column (250 mm X 60 mm, 5μm) preceded by a guard column and used a 168 diode array detector. The eluent was a linear gradient of acetonitrile (ACN), water, and 0.01% Trifluoroacetic acid (TFA) at a
flow rate of 0.8 ml/min. ACN was the A solvent in our system and water/ TFA mixture represented the B solvent. The linear gradient system was utilized since it allowed for the best separation of the various phytoalexins which could be detected in the UV range (Table 1, Figure 8). This table corresponds with the method that we implemented in all of the following HPLC studies. This method starts off with 23% ACN and 77% H₂O/TFA at time zero. The sampling period was for 30 minutes.

**HPLC ANALYSIS OF FUNGAL EXTRACT**

*P. chalmydosporum* was collected from the potato dextrose agarose plates during the maturation process. The immature stage (white colonies) and the mature stage (olive green colonies) were isolated in order to determine any differences between the two stages. Approximately 1-2 grams of the different colonies were taken from the plate for this study. The fungal material was vortexed in a 1:1 mixture of CH₃OH and H₂O to insure optimal extraction of the organic compounds from the fungal matrix. The mixture was then sonicated for thirty minutes and mashed to make certain that all of the organics were driven off of the remaining residual agar. The mother liquor that remained after sonication was extracted with 5ml ethyl acetate (EtOAc) with the use of a separatory funnel. This organic layer was then subjected to HPLC analysis where the stilbenoid like compounds were detected in the UV range at 260 and 306nm. The same process was used for both the mature and immature stages of development.

**EXUDATE COLLECTION FROM INFECTED GRAPEVINE**

Syrah vine samples were cleaned and sectioned before they could be inspected for Black Goo. The vine sections were cut into 4-5 inch segments and placed into Petri dishes which were partially filled with water to allow for sufficient xylem flow. Each
Table 1. Gradient method for the HPLC analysis in two minute increments

<table>
<thead>
<tr>
<th>Eluent flow (min)</th>
<th>%A (ACN)</th>
<th>%B (H₂O, 0.01% TFA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>23.0</td>
<td>77.0</td>
</tr>
<tr>
<td>2</td>
<td>26.0</td>
<td>74.0</td>
</tr>
<tr>
<td>4</td>
<td>30.0</td>
<td>70.0</td>
</tr>
<tr>
<td>6</td>
<td>33.0</td>
<td>67.0</td>
</tr>
<tr>
<td>8</td>
<td>37.0</td>
<td>63.0</td>
</tr>
<tr>
<td>10</td>
<td>40.0</td>
<td>60.0</td>
</tr>
<tr>
<td>12</td>
<td>44.0</td>
<td>56.0</td>
</tr>
<tr>
<td>14</td>
<td>47.0</td>
<td>53.0</td>
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<tr>
<td>16</td>
<td>51.0</td>
<td>49.0</td>
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<tr>
<td>22</td>
<td>62.0</td>
<td>38.0</td>
</tr>
<tr>
<td>24</td>
<td>65.0</td>
<td>35.0</td>
</tr>
<tr>
<td>26</td>
<td>69.0</td>
<td>31.0</td>
</tr>
<tr>
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</tr>
<tr>
<td>30</td>
<td>77.0</td>
<td>23.0</td>
</tr>
</tbody>
</table>
Figure 8. Steve-6 method.
section was examined periodically for evidence of an amber exudate which was removed with a sterile glass pipet. The exudate was dissolved in anhydrous methanol for HPLC analysis.

**TLC STUDY OF EXUDATE**

TLC of the crude exudate was carried out using a 25:3 mixture of chloroform (CHCl₃) and anhydrous CH₃OH. On a Bakelite silica plate GF, TR was spotted as a standard against the exudate sample for development (Figure 10). The TLC plate was then dried and placed in an iodine chamber. The exudate appeared to have three distinct spots (Rf values of 0.28, 0.2, and 0.1).

**HPLC ANALYSIS OF EXUDATE**

The crude fungal exudate was collected from the grapevine sections with a glass pipet and placed in anhydrous methanol for direct analysis. 20 μl samples of this crude exudate solution were injected into the HPLC and allowed to separate for 30 minutes using the C18 reversed phase column. Several aromatic stilbene-like constituents were observed in the crude exudate. Pezet and Jeandet have described the location of certain phytoalexins found in grapevines infected with *B. cinerea* (22,26). Our experimental conditions practically mirrored those that were developed by these researchers. Each major peak (13.60 min, 16.80 min, and 17.76 min) was collected and analyzed via MS.

**MS INSTRUMENTATION**

Dried sample was resuspended in 20 μl 50% Acetonitrile, 0.1% Formic Acid, 0.005% heptfluorobutyric acid and 20 μl loaded into an electrospray PicoTip™ 2μm glass emitter with infusion directly into the nanospray source of a Finnigan LCQ™ Deca
XP (ThermoElectron, San Jose, CA). The instrument was run in manual collection mode using normalized collision energy of 30, an activation Q of 0.250 with minimum full scan signal intensity at $5 \times 10^5$ and a minimum MS$^2$ intensity at $1 \times 10^4$.

**SYNTHESIS OF ε-VINIFERIN**

Pure TR was obtained from Sigma Chemical (St. Louis, MO). ε-viniferin (1,3-benzenediol, 5-[(2R,3R)-2,3-dihydro-6-hydroxy-2-(4-hydroxyphenyl)-4-[(1E)-2-(4-hydroxyphenyl)ethenyl]-3-benzofuranyl]) was obtained by replicating the experiment described by Yao et al. in 2004 (24). Yao et al. used a solution of FeCl$_3$$\cdot$6H$_2$O (1.24 g, 4.58 mmol) in water (8 ml) and which was added dropwise to a stirred solution of TR (1.03 g, 4.52 mmol) in methanol (10 ml) (24). The reaction was stirred at room temperature for approximately 49 hours. The methanol was evaporated using N$_2$ gas at room temperature and the resulting residue was diluted with water and then extracted three times with EtOAc. The combined organic layers were then concentrated under N$_2$ gas and subjected to HPLC analysis using a C-18 reversed phase column and the ACN/H$_2$O/TFA gradient.

**SYNTHESIS OF δ-VINIFERIN**

δ-viniferin (1,3-benzenediol, 5-[(1Z)-2-[2S,3S)-3-(3,5-dihydroxyphenyl)-2,3-dihydro-2-(4-hydroxyphenyl)-5-benzofuranyl]ethenyl]) was obtained by following the synthesis described by Sako et al. in 2004 (25). An equimolar mixture of TR (9.2 mg, 0.04 mmol) and silver acetate (AgOAc) from Sigma Aldrich (St. Louis, MO) (99% pure; 6.8 mg, 0.04 mmol) in dry MeOH (2.0 ml) was heated at 50°C for one hour (25). The reaction mixture turns an ashy gray color and leaves a silver coating on the glass wall as the reaction progresses. The reaction mixture was filtered using a semi-prep micron filter
to remove residual silver and the mixture was concentrated under N$_2$(g). The concentrated sample was then analyzed via HPLC using a C18 reversed phase column and the ACN/H$_2$O/TFA gradient. Three peaks were observed in the HPLC reaction of the mixture. Each fraction was then collected by multiple injections for MS analysis.

**MS OF FRACTIONS FROM SYNTHESIS OF δ-VINIFERIN**

It would appear that Sako et al. did not fully analyze the products of this AgOAc reaction. We were able to separate the mixture into three compounds using HPLC; whereas, they were only able to see one peak using TLC densiometry. Each of these compounds from our reaction scheme was collected for MS analysis. These fractions were concentrated using N$_2$(g) to evaporate off the ACN. The residue from each fraction was stored under N$_2$(g) at 5°C until they could be analyzed by LC/MS. Each of the major fractions were analyzed for their parent ion and fragmentation pattern in order to help elucidate their chemical structures.

**REACTION OF δ-VINIFERIN WITH AgOAc**

Data from the δ-viniferin synthesis showed that this viniferin has a retention time of 17.76 min. Repeated separations were carried out and combined in order to obtain a pure fraction of the δ-viniferin. The ACN was removed under N$_2$(g) and the residue was then re-suspended in methanol (2ml) and AgOAc (6.8 mg/0.04mmols) was added and stirred for 1hr. Once the reaction was complete, the sample was filtered with a semi-prep micron filter and then was subjected to HPLC analysis.

**MS OF THE REACTION OF δ-VINIFERIN WITH AgOAc**

Using AgOAc in CH$_3$OH, the 17.76 minute peak of δ-viniferin was fully converted to a peak at 18.50 minutes. This peak was not described by Sako et al. in their
original work. This fraction was collected by repeated HPLC fractionation for MS analysis. The MS data for this 18.50 min peak shows a parent ion of m/z 907.

PHOTOLYSIS OF ε, δ, AND THE 18.50 MINUTE FRACTION

Using an ultraviolet products Inc. chromato-vue model CC-20 photochamber, stilbenes, such as TR, are known to isomerize when subjected to UV light. The ε and δ viniferin standards that were isolated via HPLC were photolyzed at 260nm light for an hour. The photolyzed material was then subjected to HPLC in order to detect any isomerization of either stilbene and to establish the retention times of the cis isomers with regards to retention time. The 18.50 minute peak was also photolyzed at 260nm light for an hour.

FURTHER ANALYSIS OF CRUDE FUNGAL EXUDATE

Standard solutions (TR, δ, and the 18.32 min peak) were spiked into samples of the fresh Black Goo exudate. The objective of this experiment was to determine if there was any peak enhancement in the Black Goo exudate by these compounds. About 1ml of each standard concentration were individually added to the total volume of the exudate sample. The spiked sample was vortexed for 5 min and was then analyzed via HPLC; using the ACN/H₂O/TFA gradient method for 30 min runs. The HPLC of the spiked sample was then compared with the original HPLC of the exudate to detect if there was any peak enhancement.
RESULTS AND DISCUSSION

Black Goo has now been detected in Virginia grapevines following the examination of grapevines from Horton Vineyards located in Orange, Virginia. Our major objective was to study these infected grapevines in order to learn more about the disease, its chemical composition, and its possible mechanism of action. According to Morton, evidence from her recent trips to vineyards in America, Australia, and New Zealand has shown that many vines between the ages of one and seven years old are dying from young vine decline (27).

Our study used Syrah (3309) grapevines that were four to five years of age since young vine decline typically affects grapevines between the ages of one and seven. Each vine sample was sectioned in order to confirm that the vine was actually infected with Black Goo. The harvested vines showed signs of stunted growth and chlorosis of the leaves which is indicative of the effect of this disease on the vine. The infected vine showed evidence of a black amber gummosis that exuded from the xylem of the sectioned vine. This exudate was collected from sectioned vines that were stored in Petri dishes which had a sufficient amount of water to allow for exudate flow through the xylem. The actual chemistry of this Black Goo exudate has not been described to date. Since the Black Goo is an apparent sign of the presence of this disease in the vine, a better approximation of its chemical composition may help us understand the disease process from within the vine. This analysis has been carried out by using HPLC, TLC, and LC/MS.

All HPLC analyses were carried out using a gradient of ACN and H₂O/TFA and a C-18 reversed phase column where the most polar compounds elute out first.
The collected exudate was dissolved in anhydrous CH$_3$OH for direct injection into the HPLC and each analysis lasted 30 minutes. The HPLC analysis of the crude exudate showed three major and distinct peaks (Figure 9) labeled A (13.90 min), B (16.80 min), and C (17.76 min).

TLC of the crude exudate was carried out using a 25:3 mixture of chloroform (CHCl$_3$) and anhydrous CH$_3$OH. On a Bakelite silica plate GF, TR was spotted as a standard against the exudate sample for development (Figure 10). The TLC plate was then dried and placed in an iodine chamber. The exudate appeared to have three distinct spots (Rf values of 0.28, 0.2, and 0.1). One of these spots matched up with the TR standard. The discovery of the TR in this TLC analysis led us to carry out a spiking experiment in the HPLC. A sample of crude exudate was spiked with a sample of the TR standard (Figure 11) to detect any peak enhancement at the 13.90 minute peak. The peak enhancement confirms that TR is a major compound of the crude Black Goo exudate (Figure 12). The other two components (16.80 and 17.76 min peaks) could not be identified using TLC.

The two peaks (B and C) in the HPLC of the crude exudate were fractionally collected for analysis via LC/MS. These fractions were concentrated with N$_2$(g) and kept in ACN and refrigerated before analysis. Our analysis was done at Eastern Virginia Medical School’s Department of Proteomics. The MS for each fraction (Figures 13, 14) both showed parent ions of 455.1 m/z. This indicates that both peaks have the same molecular weight and suggest that they are isomeric. Peak A has been identified by HPLC spiking experiments and by TLC as TR. TR has a molecular weight of 228.1 g/mol which suggests that peaks B and C (both with a m/z of 455.1) may be dimers of
Figure 9. This is HPLC data of the crude exudate. This analysis was carried out using the Steve-6 method for 30 minutes. Peak A separated out at 13.90 minutes, B at 16.80 minutes, and C at 17.76 minutes.
Figure 10. TLC of the Black Goo exudate (B) spotted against standard TR (A).
Figure 11. This is an HPLC of standard TR using our ACN/ H₂O/ TFA method.
Figure 12. This shows the HPLC data of exudate after being spiked with TR.
Figure 13. MS/MS of peak B.
Figure 14. MS/MS of peak C.
TR. The presence of TR in the crude exudate is one of the most important discoveries of our research, and it has not been reported in the literature to date. TR is a well known phytoalexin that is synthesized by the vine as a result of UV irradiation or microbial stress. TR may also be presumed to be a precursor for the formation of plant viniferins, which can be dimers of TR. Langcake and Pryce discovered viniferins in *Vitis vinifera* and determined that they possessed exceptional phytoalexin properties. These authors were the first to discover that ε-viniferin has a parent ion of 454 m/z (Figure 15) (15). This parent ion is similar to the m/z of peaks B and C that were detected in the crude exudate. Pezet et al. also found that δ-viniferin was a major component discovered in downy mildew. These researchers discovered that the parent ion for this compound is also 454m/z and is considered to have originated from the dimerization of TR in the plant (21). Based on our preliminary MS data, there is a strong possibility that TR (Peak A) may be acting as a precursor to the formation of peaks B and C which may be the ε and δ viniferins.

*P. chalmydosporum* was collected directly from the woody portion of the Syrah grapevine segment. This organism was cultured on potato dextrose agar in order to induce colony formation. Positive identification of the fungus was achieved by following the guidelines that were established by Mugani. According to Mugani, the fungus is initially shiny white and elevated on the plate which progressively changes to an olive green color as the fungus matures (period of a week) (23). Our fungus shared the same morphological properties (i.e. color, shape, etc.) that Mugani reported (23).
**Figure 15.** MS/MS of ε-viniferin standard from Pezet et al. (22).
Fungal analyses were conducted at the immature (white) and mature (green) stages. Fungal colonies from each stage were extracted into a 1:1 mixture of CH$_3$OH/H$_2$O. These fungal mixtures were sonicated at room temperature in order to make certain that most of the organics were extracted from the agar. The mother liquor that remained following each sonication was filtered and then extracted with EtOAc. Each of the organic layers were concentrated by evaporation under N$_2$(g) and analyzed by HPLC.

The HPLC analyses of the extracts from both the immature and mature fungal stages showed that this extract exhibited the same peaks as those that were detected in the Black Goo exudate. The HPLC of the immature stage showed a peak at 13.90 min which corresponds to the TR peak found in the Black Goo exudate (Figure 16). This fungal extract was then spiked with standard TR to demonstrate peak enhancement. The peak at 13.90 min was indeed enhanced which confirms that TR is present in both the fungal extract and exudate (Figure 17). TR was found in greater amounts during the immature stage when compared to the mature stage. HPLC data of the mature stage of the fungus showed peaks at 16.80 and 17.76 min, where the 16.80 min peak was the most intense (Figure 18). This observation was quite surprising because the same peaks were detected in the Black Goo exudate. The Black Goo exudate appears to have an intriguing relationship with the *P. chalmydosporum* fungus. Based on these results, it is possible that the exudate is actually produced by the fungus which is then released and accumulates in the xylem of the infected grapevine. This obstruction of the xylem flow by these high molecular and water insoluble metabolites could account for the development of the young vine decline disease.
Figure 16. HPLC of the fungal extract during its immature stage.
Figure 17. HPLC of fungal extract spiked w/ TR.
Figure 18. HPLC of the fungal extract during its mature stage.
We have compared our HPLC analyses of the exudate and the fungal extract with the recent work of the Jeandet and Adrian groups where the groups used similar HPLC conditions to those established in our work (29,30). Several of these compounds were described as the ε and δ viniferins which are apparently oxidative coupling by-products in the grapevine. Some of there same peaks have been observed in our study. In view of the peak similarity in the HPLC, we sought to develop methods for synthesizing these viniferins as standards for a more complete HPLC identification.

Yao et al. described a chemical reaction for the production of ε-viniferin (Figure 19) (24). We were interested in reproducing this reaction in order to secure this viniferin as a standard for HPLC analysis. This reaction used aqueous ferric chloride which was added to a solution of TR and CH₃OH while being stirred at room temperature for 49 hours. The solvent was evaporated off of the organic material with N₂(g) after the reaction was complete, then this organic residue was extracted with three portions of EtOAc. Each of the layers were combined together and concentrated for HPLC analysis.

The HPLC analysis for the production of ε–viniferin by this technique was rather complex, however, Yao et al. established that ε–viniferin has a retention time close to 16.80 min (24). The HPLC of our synthetic product showed a peak at 16.88 min but also exhibited a peak at 13.84 min that corresponded to TR (Figure 20). This suggests that this reaction is not very efficient and that there is a major portion of un-reacted TR. Yao et al. reported that ε–viniferin was not able to be produced in quantitative yield, however, this reaction has now given us the capability of synthesizing this viniferin standard. This group used the similar conditions when compared to the parameters of our HPLC. Based on our HPLC analysis of both the fungal extracts and the Black Goo
Figure 19. Synthesis of ε-viniferin. (Yao et al.).
Figure 20. Our HPLC data from the Yao et al. reaction. The 16.88 min peak is identified as ε-viniferin.
Figure 21. This is the mechanism that Yao et al. proposed. The A. denotes the resorcinol free radical (identical to the one shown above).
exudate, it would appear that ε-viniferin is a major component in both.

The ε–viniferin mechanism proposed by Yao et al. is a oxidative coupling reaction involving Fe\(^{3+}\) as the oxidant (Figure 21) (24). This reaction involves intermolecular resorcinol coupling of TR. The resorcinol group of the TR molecule first loses a Hydrogen to produce the resorcinol radical and will quickly react with another molecule of TR at the alkene double bond as a result (24). After 49 hrs, the reaction appears not to proceed to full conversion of ε–viniferin, however, we have been able to produce enough of it so that we could use it as an effective standard. We have therefore identified both peaks A as TR and B as ε–viniferin.

The synthesis of δ-viniferin has been reported by Sako et al. and we have replicated this synthesis in order to isolate this viniferin for HPLC comparison with Black Goo exudate. Sako reported that δ-viniferin was produced in nearly quantitative yield by using AgOAc as the oxidant (25). The reactant TR and AgOAc were suspended in methanol and stirred at 50 °C for 1 hr (Figure 22) These researchers have proposed a mechanism for the synthesis of δ-viniferin (Figure 23) This mechanism is described as a free radical oxidative dimerization involving phenolic coupling. The silver ion appears to be selective for phenolic coupling since none of the ε –viniferin was produced in the reaction. This mechanism starts with the oxidation of the phenolic group of TR which yields a resonance stabilized free radical. We have repeated this experiment a number of times and find that the reaction proceeds to give the desired δ-viniferin but there is also a second product that was not repeated by Sako et al. The δ-viniferin has been isolated via HPLC and shown to have a retention time of 17.76 min, which is the same as that of peak C in the Black Goo exudate. MS analysis of this peak gives a parent ion of 455 m/z just
Figure 22. Synthesis of δ-viniferin. (Sako et al.).
Figure 23. Mechanism proposed by Sako et al. for the synthesis of δ-viniferin.
Figure 24. δ-viniferin MS/MS from our AgOAc reaction. (Sako et al.)
Figure 25. MS/MS of the 18.50 min peak from our AgOAc reaction (Sako et al.).
like the parent ion that was detected in peak C of the exudate (Figure 24). The second peak in our HPLC analysis (18.50 min retention time) increases in intensity as the reaction proceeds and has a parent ion of 907 m/z (Figure 25) (32).

The HPLC for the end product of this reaction showed that there were three major peaks at the beginning of the reaction and then two major peaks after a substantial period of mixing (Figure 26). Figure 26 depicts that the final product of the reaction is a mixture and this was not reported by Sako et al. Our HPLC shows that there is an intense TR peak which separated at 14.08 min at the start of the reaction. After a little over one hour, the TR levels decreased and the peaks of 17.78 and 18.50 min both increased in intensity. The detection of the 18.50 min peak was first discovered by our group. Sako et al. subjected their product to silica gel column chromatography for purification and reported that δ-viniferin was obtained in almost quantitative yield (25). It may be that the mini column used by the Sako group was not efficient enough in separating the compounds of the mixture. The HPLC clearly showed that the reaction of TR with AgOAc is more complicated than just the “almost quantitative” synthesis of the δ-viniferin. We found this unexpected discovery intriguing since it may play a role in the Black Goo exudate chemistry.

The first task was to repeat the Sako reaction using timed experiments which would be followed by HPLC. The reaction mixture was analyzed at t= 0, 0.5, 1, and 1.5 hr (Figures 27-30). The HPLC analyses showed a rapid decline in TR as the reaction proceeded over the duration of the reaction. As the TR levels decrease, their two distinct peaks appear in the reaction mixture. The peaks are fairly non-existent during the early stage of the reaction whereas the TR levels are high. Based on our results, TR is
Figure 26. HPLC of our Sako et al. reaction.
presumed to be a precursor to these two peaks. One of these peaks has already been identified as δ-viniferin and the other has not yet been fully classified.

The fractions that we have collected from the Sako et al. reaction has provided us with two samples that we could use for spiking the Black Goo exudate samples. The Black Goo exudate HPLC (Figure 9) showed peaks at 16.80, 17.76, and peaks in the 18-19 min range. About a ml of each fraction was added to exudate samples for HPLC analysis. The HPLC of these spiked samples were compared to the HPLC from figure 9. The spiked samples did show that there was peak enhancement at both 17.76 and 18.50 min (Figures 31-32). Thus, our results suggest that δ-viniferin and the 18.50 min mystery peak are major components of Black Goo. It is now apparent that viniferins are naturally synthesized within the vine that is infected with P. chalmydiosporum.

The 18.50 min peak that we observed from the Sako et al. reaction may be a dimer of δ-viniferin since the peak has a parent ion of 907 m/z (930.9 m/z w/ sodium ion). This observation has not been reported to date and needs to be examined further. The first task was to obtain enough of the δ-viniferin standard so that it could be used with TR and AgOAc to determine if the viniferin undergoes further oxidative dimerization to form the 18.50 min peak. In using HPLC, we have shown that δ-viniferin is converted to the 18.50 min peak when reacted with AgOAc (Figures 33-35).

Photolytic experiments were carried out to determine if the peaks were able to convert from one isomer to another when in the UV. Both of the peaks, δ–viniferin and 18.50 min fraction, were photolyzed and analyzed via HPLC (Figures 36-37). Each of these figures showed that each of the peaks were possibly converted to other isomers (cis/ trans). The data suggests that the 18.50 min peak may be a compound that has a double bond due to its conversion from 18.50 min to 19.6 min while analyzing the photolyzed
Figure 27. HPLC of our AgOAc reaction at $t=0$ min.
Figure 28. HPLC of our AgOAc reaction at $t = 30$ min.
Figure 29. HPLC of our AgOAc reaction at $t = 1$ hr.
Figure 30. HPLC of our AgOAc reaction at $t = 1.5$ hr.
Figure 31. HPLC of δ-viniferin spiked into the Black Goo exudate.
Figure 32. HPLC of the 18.50 min peak spiked into the Black Goo exudate.
Figure 33. HPLC of 8-viniferin fraction.
Figure 34. 18 min peak from the dimerization reaction of δ-viniferin and AgOAc.
Figure 35. HPLC of 18 min peak from the Sako et al. reaction.
sample in the HPLC. The possible structure for the tetramer is diagramed in figure 38. The compound is proposed to be formed due to the δ-viniferin being able to continue to undergo oxidative dimerization through phenolic coupling while in the presence of AgOAc.
Figure 36. HPLC of the δ-viniferin photolyzed in the UV.
Figure 37. HPLC of the 18.50 min peak photolyzed in the UV.
Figure 38. Proposed structure for the 907 m/z tetramer from the Sako et al. reaction.
CONCLUSION

The results and data that we have accumulated regarding the young vine decline and the presence of the Black Goo exudate in the infected grapevine has provided us with some insight into the origin, the chemical nature, and the possible mechanism of action for viniferin formation as it relates to the development of the disease. This research has now proven for the first time that the disease known as young vine decline (Black Goo) has arrived and is present in one of Virginia’s vineyards and is likely present in many of the other 120 vineyards in the state. The Black Goo infection that we and others have seen throughout the country can in most cases be associated with contaminated root stock. The Syrah vines that were used in our study all exhibited Black Goo exudate when sectioned and the *P. chalmydosporum* fungus was isolated from the same vines. The fungal cultures used in this study all exhibited the classic or characteristic morphology (i.e. shape, color, growth pattern) as the *P. chalmydosporum* that was described in the literature (23).

Our HPLC and MS studies of the Black Goo exudate have shown that this marker for the young vine decline is in fact a complex mixture of three phytoalexins including TR, δ, and ε viniferins together with some yet unidentified high molecular weight compounds. Perhaps the most interesting aspect of our studies has shown that a similar analysis of the *P. chalmydosporum* fungus itself contains these same three phytoalexins which suggested that the fungus is in fact the origin of the Black Goo in the grapevine. This might suggest that the ε and δ viniferin identified by us in the Black Goo exudate are in deed metabolites of the *P. chalmydosporum* fungus. Because of their low solubility and high molecular weight, these compounds when exuded from the fungus
would accumulate in the xylem and produce the obstruction of the flow of vital nutrients for the vine, leading to young vine decline (33).

Our research has led us to believe that there is a close relationship between Black Goo and a similar fungal infection of grapes known as *Botrytis cinerea*. In a 2002 paper, Kan et al. suggested that *B. cinerea* infection of grapes induces a type of self intoxication in the plant which ultimately leads to a more agreeable environment for further fungal infection of the host (34). The *B. cinerea* scenario can be pictured as arising from specific responses to the fungal infection (Figure 39). The *B. cinerea* infection first leads to an increase in TR levels in the grapes. This enhanced TR level then triggers a genetic component in the *B. cinerea* laccase enzyme to effectively initiate phenolic coupling of TR to produce the ε and δ viniferin observed in the infected grapes. These viniferins are considered to be fungitoxic, thus, leading to a slow down of further *B. cinerea* infection and thus creates the concept of self intoxication. As the grapes mature, the level of TR decreases naturally which leads to a decrease in viniferin synthesis. This scenario then allows the fungus to readily infect the grapes since the level of fungitoxic viniferins has been controlled. Thus, the *B. cinerea* infection becomes dependent on the continued synthesis of the viniferins until the conditions (low viniferin levels) are more agreeable or conducive to further infection. It creates a self protective TR based mechanism for its survival and increased fungal growth and infection.

Based on our results, we would suggest that the *Phaeoacremonium chalmydosporum* fungus (Black Goo fungus) may be operating in a very similar function. We have demonstrated that the fungus and the Black Goo exudate both contain TR and ε and δ viniferin. One would assume that the Black Goo fungus also contains the
Figure 39. *B. cinerea* infection scenario.
necessary laccase needed to convert TR into these fungitoxic viniferins. The presence of the polyphenolic components suggests that the Black Goo fungus may also be creating a self intoxication scenario in order to produce a more agreeable environment for its infective mechanism. The release of the viniferins into the xylem might be seen as an indirect mechanism that would lead to a weakening of the plant resistance and the conditioning of the plant material for easier infection. Unfortunately, this Black Goo scenario leads to young vine decline and the eventual death of the vine. Black Goo has been described as a pioneering disease that effectively pre-conditions the vine to a more devastating fungal infection mechanism known as Esca. The intriguing aspect of the Black Goo scenario is that the fungus is apparently capable of synthesizing TR, presumably via a Black Goo laccase mechanism. The synthesis of TR within the fungus might be explained on the basis of the need for TR for eventual viniferin synthesis that would lead to self intoxication.

The *B. cinerea* scenario described in infected grapes accounts for the increased levels of TR as a result of this fungal stress. With the Black Goo fungus, there is no direct mechanism for TR synthesis in the xylem, thus the fungus has developed its own TR synthesis with the aid of the laccase is able to complete the scenario by producing the fungitoxic viniferins. The release of these fungal metabolites is simply a marker or initiator of Black Goo infection. Unfortunately, the Black Goo exudate leads to further complications with the response to xylem flow and vine health.

We have focused to some extent on the synthesis of suitable viniferin standards to help identify the peaks in the Black Goo exudate and the fungal extract. The synthesis of the ε-viniferins following the work of Yao et al. using FeCl₃ was successful but gave
rather poor yield of the desired coupling product (24). The synthesis of the \( \delta \)-viniferin according to the method of Sako et al. was more successful and provided us with an authentic \( \delta \)-viniferin sample which was used in our HPLC studies to positively identify the peak at 17.30 min in the Black Goo exudate (25).

We have shown that this synthesis was not fully explored and that there is indeed a second oxidative coupling product. This new type of viniferin apparently arises from the AgOAc coupling of \( \delta \)-viniferin to produce a dimer (m/z 907). Interestingly enough, this new dimer of \( \delta \)-viniferin appears at 18.30 min in our HPLC studies and there is evidence that this same material is present in very similar levels in the Black Goo exudate. The presence of a TR tetramer would seem feasible in view of the oxidative coupling capacity of the fungal laccase to produce both the \( \varepsilon \) and \( \delta \) viniferins.
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VITA

David Michael McGinnis

Education
2005  M.S. Degree in Chemistry.
      Department of Chemistry and Biochemistry, Old Dominion University,
      Norfolk, Virginia 23529- Principal Investigator, R. L. Williams.
2002  B.S. Degree in Biochemistry.
      Department of Chemistry and Biochemistry, Old Dominion University,
      Norfolk, Virginia 23529.
      Tidewater Community College, Virginia Beach, Virginia 23453.

Employment
2005  Research Assistant.
      Enological Research Laboratory, Department of Chemistry and
      Biochemistry, Old Dominion University, Norfolk, Virginia 23529.
2004  Graduate Teaching Assistant.
      Department of Chemistry and Biochemistry, Old Dominion University,
      Norfolk, Virginia 23529.
2003-2002 Chemist
      Research Laboratory, K&M Environmental, Inc., Virginia Beach,
      Virginia 23429.
2001 Undergraduate Teaching Assistant
      Department of Chemistry and Biochemistry, Old Dominion University,
      Norfolk, Virginia 23529.

Papers, Presentations, and Awards
2005  Outstanding Teaching Assistant Award
      Department of Chemistry and Biochemistry, Old Dominion University,
      Norfolk, Virginia 23529.
2005  Virginia Academy of Science 83rd Annual Meeting.
      “Synthesis of Antifungal Viniferins; Their Relationship to Young Vine
      Decline” D. M. McGinnis (presenter), R. L. Williams, and Old
      Dominion University.
2004  Virginia Academy of Science 82nd Annual Meeting.
2004  American Journal of Enology and Viticulture.
      “Chemical Nature of Black Goo in Young Vine Decline” R. L.
      Williams, D. M. McGinnis, and Old Dominion University.
2002  Virginia Academy of Science 80th Annual Meeting.