A DECISION TOOL AND MOLECULAR TYPING TECHNIQUE FOR A PLANT PATHOGEN FORENSIC APPLICATION, USING WHEAT STREAK MOSAIC VIRUS AS A MODEL PATHOGEN

By

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

CHAPTER III

CHAPTER IV

CHAPTER V

CHAPTER VI

LIST OF FIGURES

Figure Page

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER V

CHAPTER VI

APPENDICES

CHAPTER I

INTRODUCTION

The terrorist attacks of 2001 on the United States spurred an assessment of the vulnerabilities that exist in the U.S. to similar attacks; the majority of the vulnerabilities identified were in the agricultural sector (Cupp et al, 2004). Many naturally occurring threats, including an abundance of plant diseases, affect agriculture. Approximately 14% of crops worldwide are lost due to pathogens, including bacteria, fungi, viruses, and nematodes (Agrios, 2005). Some of these pathogens may be easily obtainable from the environment and manipulated for use as bioweapons (Bronze et al, 2005).

Should a plant pathogen be used as a biological agent in an attack on our agriculture, the strength and stability of our country may be affected. The partial or complete loss of a crop or animal system may not only reduce or eliminate a food item for human consumption, but may also affect foodstuff for livestock or pets and could trigger trade embargoes to be enacted leading to a significant reduction in the Gross Domestic Product (GDP) (Cupp et al, 2004; Fletcher et al, 2006; Osborne, 2005; Wheelis et al, 2002). Consumer prices may increase, and in extreme cases could result in social turmoil and the loss of public faith in the US government to protect the nation (Wheelis et al, 2002). The U.S. Government has determined that because such significant impacts could result from an attack, it is important that the U.S. prepare for such an event to assure rapid, efficient, and organized responses to minimize damage (Bush, 2004).

Due to the prevalence of plant disease that occurs naturally, an intentional release of a pathogen may go undetected (Fletcher et al, 2006). When a producer suspects an unhealthy crop, a Cooperative Extension Agent is typically the first person contacted. If disease is suspected, a

sample may be sent to a diagnostic lab, often associated with the National Plant Diagnostic Network (NPDN), for identification of the disease and causal agent (Stack et al, 2006, 2007; Stack and Fletcher, 2007). The extension and diagnostic lab personnel are often the first line of defense against an intentional introduction and must be aware of signs that may suggest such an event has occurred.

After an intentional introduction is detected and a response is underway, an investigation to identify the source of the introduced pathogen must occur. Being prepared with the resources needed to attribute an intentional biological attack on plant resources will require merging aspects of plant pathology and forensic science. Multiple "gaps" exist in the field of plant pathogen forensic science, including lack of specific forensic methods, forensically-trained personnel, targeted research, adequate facilities, and funding (Cupp et al, 2004; Fletcher et al, 2006; Wheelis et al, 2002). Current microbial forensic identification methods must be validated and new methods developed that are more specific, sensitive, and efficient. Molecular typing methods are needed to discriminate among samples (Fletcher et al, 2006). Research priorities also include understanding pathogen genetic characteristics, evolution and phylogeny, and mutation rates (Fletcher et al, 2006). Forensic investigations require specific and consistent procedures and protocols and careful avoidance of contamination. Appropriate strategies for handling and storing diseased plants and plant materials are needed (Budowle et al, 2006; Fletcher et al, 2006).

Tracing back a pathogen to its original source is important for forensic investigations and also epidemiological and phylogenetic studies. Satellite imagery and geographic positioning systems (GPS) are technologies that have recently been adopted in plant pathology for identifying the foci of a disease outbreak and monitoring the spread over time (Maffei and Arena, 1992; Nilsson, 1995; Pozdnyakova et al, 2000). Using molecular techniques, genetic variation of the pathogen can be determined and correlated to geographic location. While many of these techniques, such as sequencing, restriction fragment length polymorphism (RFLP), and multi-

locus variable number tandem repeats analysis (MLVA), provide adequate information to perform such an analyses, the procedures can be costly, labor intensive, and may have limitations as to the condition of the nucleic acid template required for successful analysis (Budowle et al, 2005; Vincelli and Tisserat, 2008; Keim et al, 2000, 2004). To provide a rapid, cost effective, and simple technique for use in a forensic investigation, other techniques should be explored, such as using single nucleotide polymorphisms (SNPs) to create a genetic profile of the pathogen (Budowle et al, 2005).

Research efforts are underway to address existing gaps. For example, a method for molecular typing of *Wheat streak mosaic virus* (WSMV) by SNP analysis was adapted recently from protocols designed originally to test human DNA in forensic applications (Carver, 2007). By comparing three strains of WSMV (OSU, 964, and Plant A-D), three SNP sites were identified in the CP region. After viral isolation/purification, cDNA amplification, and ddNTP attachment, the products were separated using capillary electrophoresis. Each strain had a unique pattern of electropherogram peaks, demonstrating the discriminatory power of this new method (Carver, 2007). These patterns were consistent on repeated runs, not only for the nucleotides themselves but also for the ratio of the nucleotides present suggesting not only qualitative, but also quantitative results may be achieved using this technology (Carver, 2007).

In this research, I investigated the aspects of a WSMV outbreak in wheat to identify elements that could be employed to elucidate whether an intentional introduction of the virus may have occurred. I also utilized knowledge of WSMV evolution and genetic characteristics to explore and validate a method that could be useful in forensic attribution of a biocrime. Specific objectives of this research follow:

1. Develop a decision tool, a series of questions and guidelines to assist law enforcement personnel in determining whether a plant disease outbreak was natural or human-incited.

2. Enhance and validate the use of single nucleotide polymorphisms (SNP) for molecular WSMV strain discrimination for forensic applications.
3. Determine whether WSMV SNP profiles are altered by common field treatments applied to wheat during the growing season.
4. Evaluate the use of the SNP typing method to rapidly and efficiently analyze the spatial spread of WSMV.

LITERATURE CITED

Agrios, G. E. 2005. *Plant Pathology* (5th ed.). Oxford, UK: Elsevier Academic Press.

Bronze, S.B., and Greenfield, R.A. 2005. Introduction, p. 1-23. *In* M.S. Bronze, R.S. Greenfield (ed.), Biodefense: Principles and Pathogens. Horizon Bioscience, Norfolk, England.

Budowle B., Johnson M.D., Fraser C.M., Leighton T.J., Murch R.S., and Chakraborty R. 2005. Genetic analysis and attribution of microbial forensics evidence. Crit. Rev. Microbiol. 31:233-254.

Budowle, B., Schutzer, S. E., Burans, J. P., Beecher, D. J., Cebula, T. A., Chakraborty, R., Cobb, W. T., Fletcher, J., Hale, M. L., Harris, R. B., Heitkamp, M. A., Keller, F. P., Kuske, C., LeClerc, J. E., Marrone, B. L., McKenna, T. S., Morse, S. A., Rodriquez, L. L., Valentine, N. B., and Yadev, J. 2006. Quality sample collection, handling, and preservation for an effective microbial forensics program. Appl. Environ. Microbiol. 72:6431-6438.

Bush G.W. 2004. Homeland Security Presidential Directive 10: Biodefense in the 21st Century. The White House.

Carver, J. 2007. Molecular typing of Wheat streak mosaic virus for forensic applications. M.S. Thesis. Oklahoma State Univeristy-Center for Health Sciences, Tulsa, OK.

Cupp, O. S., Walker II, D. E., and Hillison, J. 2004. Agroterrorism in the US: Key security challenge for the 21st century. Biosecur. Bioterror. 2:97-105.

Fletcher, J., Bender, C., Budowle, B., Cobb, W. T., Gold, S., Ishimaru, C., Luster, D., Melcher, U., Murch, R., Scherm, H., Seem, R. C., Sherwood, J. L., Sobral, B. W., and Tolin, S. 2006. Plant pathogen forensics: capabilities, needs, and recommendations. Microbiol. Mol. Biol. Rev. 70:450-471.

Keim P., Price L.B., Klevytska A.M., Smith K.L., Schupp J.M., Okinaka R., Jackson P.J., and Hugh-Jones M.E. 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J. Bacteriol. 182:2928-2936.

Keim P., Van Ert M.N., Pearson T., Vogler A.J., Huynh L.Y., and Wagner D.M. 2004. Anthrax molecular epidemiology and forensics: using the appropriate marker for different evolutionary scales. Infect. Genet. Evol. 4:205-213.

Maffei H.M., and Arena J.V. 1992. Forest insect and disease risk and occurrence maps in GIS: application to integrated resource analysis, Proceedings: Spatial analysis and forest pest management, Virginia. pp. 8-14.

Nilsson H.E. 1995. Remote sensing and image analysis in plant pathology. Ann. Rev. Phytopathol. 33:489-527.

Osborne, D. J. 2005. A short history of warfare emphasizing the biological aspects. In J. I. Cooper, T. Kuehne and V. P. Polischuk (Eds.), Virus Diseases and Crop Biosecurity (pp. 1-8). Dordrecht, The Netherlands: Springer.

Pozdnyakova L., Oudemans P.V., Hughes M.G., and Gimenez D. 2000. Estimation of spatial and spectral properties of phytophthora root rot and its effects on cranberry yield, 2nd International Conference on Geospatial Information in Agriculture and Forestry, Florida. pp. 57-70.

Stack, J., Cardwell, K., Hammerschmidt, R., Byrne, J., Loria, R., Snover-Clift, K., Baldwin, W., Wisler, G., Beck, H., Bostock, R. M., Thomas, C. S., and Luke, E. 2006. The National Plant Diagnostic Network. Plant Dis. 90:128-136.

Stack, J., and Fletcher, J. 2007. Plant biosecurity infrastructure for disease surveillance and diagnostics. In I. o. Medicine (Ed.), Global infectious disease surveillance and detection:

Assessing the challenges - finding the solutions (Vol. Workshop summary, pp. 95-106). Washington, D.C: The National Academies Press.

Stack, J., Hammerschmidt, R., Hudler, G., Luke, E., and Bostock, R. 2007. National Plant Diagnostic Network: a record of accomplishment. CSREES five-year review. Retrieved from http://www.npdn.org/webfm_send/2

Vincelli P., and Tisserat N. 2008. Nucleic acid-based pathogen detection in applied plant pathology. Plant Dis. 92:660-669.

Wheelis, M., Casagrande, R., and Madden, L. V. 2002. Biological attack on agriculture: low-tech, high-impact bioterrorism. BioScience 52:569-576.

CHAPTER II

REVIEW OF LITERATURE

Between 1845 and 1850, famine swept the land of Ireland (Fraser, 2003). Field after field of potatoes rotted, leaving little crop yield for the people. This destruction was due to the oomycete pathogen *Phytopthora infestans*, the causal agent of potato late blight disease (Fry et al, 1997).

Potatoes were first introduced to Ireland from Spain in 1590 (O Grada, 1995), and became an important food staple for the Irish because their climate was proved excellent for this crop, which provided nutrition equivalent to that of corn (*Zea mays)* but at one-third the cost (O Grada, 2005). Dependency set the stage for the famine, when the usual yield of six to seven tons per acre was reduced to an average of half a ton per acre (O Grada, 2005). Soon, prices started to skyrocket, and finding food became virtually impossible for many people (O Grada, 2005). Although efforts were made to feed the hungry, for some, it was not enough. About a million people died of starvation or famine-related diseases while another million people fled the country in hopes of finding a better life (Hickey et al, 1980).

The Irish potato famine is a prime example of the vulnerability of crops and the damage that can occur if a major food staple became unavailable. The dependence on the potato crop and the appropriate environmental conditions for the pathogen together resulted in one of the

most devastating historical events (Fraser, 2003). Even though this outbreak was natural, it demonstrates that a disease intentionally spread by man also has the potential to create a disastrous outcome.

I - Bioterrorism

Bioterrorism means "the use of infectious agents or other harmful biological or biochemical substances as weapons of terrorism" (Oxford Dictionary of English, 2005). Bioweapons, such as *Bacillus anthracis*, *Shigella*, and *Salmonella*, can be used to attack humans, animals, and crops (Bronze et al, 2005). Perpetrators of bioterrorism could be foreign or domestic.

Many instances of the use of bioweapons have been reported. When biological agents are used in war, it is known as biowarfare (Bronze et al, 2005). Biocrime refers to a direct attack on a person or, for example, sabotaging a nearby farmer's crop to benefit one's own business (Bronze et al, 2005). A third area is agroterrorism, which can broadly be defined as a terrorist attack on crops and animals that are critical to the food supply (Bronze et al, 2005). The September 11, 2001 attack on the World Trade Center and the Pentagon increased the concern about terrorist attacks on many vulnerable sectors of the U.S., including agriculture (Cupp et al, 2004). The concentrated nature of crops, easily obtainable biological agents, and highly detrimental impacts make agroterrorism a potential threat.

I – a - History

Biological attacks on food production systems have occurred for centuries. In the first century BC, Carthage was a major agricultural and trading supplier of the Middle East. When the Romans defeated the Carthaginians militarily, the Romans spread salt over the land, destroying the economy of the Carthaginians. The land became a desert for more than 25 years (Osborne, 2005).

Human health was threatened when victims of the plague were hurled into the city of Kaffa, a fortress on the Crimean Coast, by the Tartars in 1346. Kaffa's defenders fled from the city instead of risking infection from the plague, making it easy for the Tartars to conquer the city (Osborne, 2005).

The first documented occurrence of bioterrorism in North America was in 1763 during the siege of Fort Pitt when blankets and bedding contaminated with smallpox scabs were given as gifts by the British forces to indigenous communities. During the Cold War years, crops were targeted for biological warfare, although the bioweapons are not known to have been deployed. By 1969, the U.S. had stockpiled an estimated 30,000 tons of wheat stem rust and Iraq was suspected of developing a wheat smut bomb (Osborne, 2005).

I – b - Impacts

Although the mortality and morbidity rates of a direct attack on humans using biological agents, such as the casual agents of anthrax, smallpox, or plague, would be significantly higher than those incurred by an agricultural attack, a deliberate attack on crops, livestock, or other food supplies could potentially cause extensive economic hardship. Food supplies could be reduced by spoilage of crops. Farmers could accrue financial damage and there could be significant decreases in exports and trade. Cost of eradication could also be a significant burden. Consumers could lose confidence in the government, causing political strife (Osborne, 2005).

Agriculture in the U.S. is an essential aspect of the economy, accounting for 11% of the gross domestic product (GDP), and 16% of U.S. jobs (Monke, 2006). The most significant economic consequence would be the loss of trade, since $50 billion/year is received from agricultural exports. Countries that are free from a certain disease can restrict trade with countries that have that disease. Billions of dollars could be lost because of such embargoes (Wheelis et al, 2002). A recent example of agricultural trade restrictions occurred in 2001 when

the United Kingdom (UK) suffered a foot-and-mouth disease (FMD) endemic. FMD is the most contagious animal disease known, with nearly 100% of the exposures resulting in infection. The European Union (EU) blocked all trade of beef, sheep, swine, and associated products from the UK, resulting in a $5 billion loss. Other losses associated with this embargo totaled over $6 billion, bringing the total loss to $11 billion (Cupp et al, 2004; Wheelis et al, 2002).

Crop quality and quantity can also be affected significantly by agroterrorism. Plant diseases incited by natural causes are estimated to cost the U.S. $33 billion per year (Madden et al, 2003). This estimate includes loss of crop yield and quality, as well as disease control measures such as pesticide application and breeding for resistance. If a field becomes infected with a plant pathogen, the cleanup of the disease can be a significant financial burden for the producer. Some pathogens can be controlled by removing infected plants, while other pathogens can be controlled only by destroying the entire crop.

The destruction of crops or livestock can also affect the consumer in the form of higher product prices. In 1999, a frost destroyed the California orange crop, resulting in inflation of orange prices as the Florida industry attempted to fill the nation's needs. Another consequence of decreased yield can be unemployment. The orange crop loss in California was followed by a significant increase in the unemployment rate. If distributors or retailers find another product source that is reliable and affordable, they may not return to their original source, resulting in significant long-term loss to the original provider (Cameron et al, 2001).

Just as the consumer can lose confidence in the industry, they can also lose confidence in the government to protect the country's critical resources. Following a biological attack, restoration of public trust may require the development of new methods of governmental response and improved prevention strategies, both of which could take years (Cameron et al, 2001).

I – c - Vulnerability

Since the September 11, 2001 attack on the World Trade Center and the Pentagon, the threat of terrorism has impacted U.S. policy. To prepare for possible future terrorist attacks, the U.S. government has identified seven vulnerable areas: transportation, telecommunications, governance, water supplies, food production, food processing and food distribution. Three of the seven are agricultural in scope, while the remaining areas are important for the agricultural industry (Cupp et al, 2004).

Agriculture is considered vulnerable not only because of its high economic value, but also because of its concentrated nature of the industry. The number of farms in the U.S. has declined from 6.3 million in 1929 to 2.2 million in 1998. The decrease in number, however, has been accompanied by an increase in size (Cupp et al, 2004). Crops are grown over large areas, making surveillance virtually impossible (Madden et al, 2003). The impact of an outbreak on the agricultural industry could be more significant for a large corporate farm than one on a small farm. For instance, a FMD outbreak is significantly different in a large cattle operation than in a small, geographically isolated herd (Goodrich et al, 2005).

Planting crops as monocultures, a common practice in developed nations, can create particular vulnerability because all plants in the field are equally susceptible to a pathogen. The 1970 epidemic of southern corn leaf blight (SCLB) in the U.S. is regarded as the most damaging and wide spread disease occurrence in the history of plant pathology and is a great example of the risk of monocultures (Tatum, 1971; Ullstrop, 1972). A new hybrid was created for corn that resulted in male seed sterility. This trait was so desirable that over 85% of the hybrid corn planted in the US was of this variety. However, the susceptibility of this variety to *Helminthosporium maydis*, the causal agent of SCLB, was unknown at the time, which was quickly realized by the rapid spread of the fungus from southern U.S. through the Corn Belt into

Canada (Levings III, 1990). In addition to the susceptibility of the variety, the rapid spread was facilitated by an unusually wet growing season and the emergence of an unknown, more aggressive race of the pathogen, known as "Race T." Unlike the old race of the pathogen, Race T causes rotting of the ears and stalk, resulting in a complete loss of the crop in many of the southern states and a 50% yield loss in many northern states. As a result, corn prices increased from $1.35/bushel to $1.68/bushel and many importers of US corn shifted to other crops for livestock feed (Ullstrop, 1972). There was an immense amount of publicity from the event creating fear among communities for future outbreaks and toxicity to livestock feeding on infected corn. Numerous conferences and meetings were held to discuss response plans and determine a solution for future crops, leading to the conclusion to label corn seed bags with the variety and planting more than one variety in each field. This decision is one of the main reasons this disease was not as severe in the following year.

There are many opportunities for a perpetrator to introduce a plant or animal pathogen into the food process, as depicted in Figures 1 and 2. Multiple "entry points" exist along the food distribution pathway at which a pathogen could enter and potentially cause an infection, leading to reduced quality and/or quantity of foodstuff. The number of entry points varies at each level because of surveillance, time duration of each level, or distance traveled to get to that point. For instance, in the U.S. meat travels an average of 1,000 miles from farm to table, with multiple entry points along the way (Cupp et al, 2004).

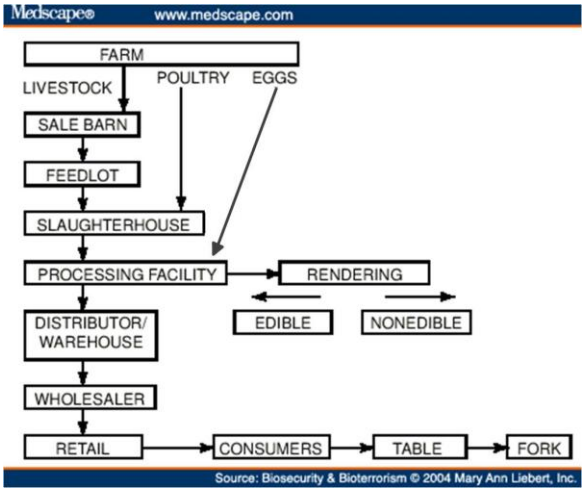


Figure 1: "Hoof to plate"; Food supply chain for animal products (modified from Cupp et al, 2004)

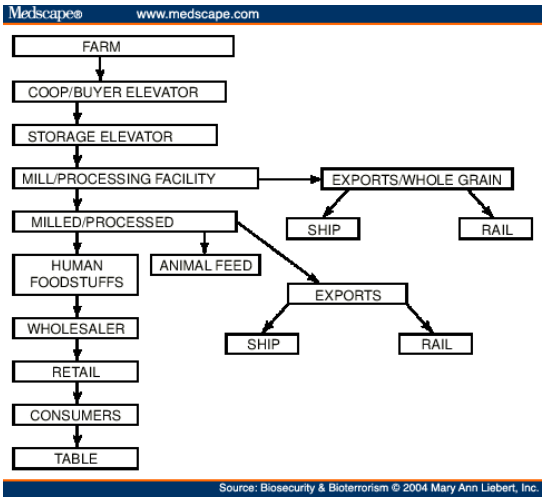


Figure 2: "Seed to plate"; Food supply chain for crops (Cupp et al, 2004)

Stockyards, processing plants and slaughterhouses have relatively little surveillance, and only a few large businesses process the majority of the meat, resulting in several vulnerable entry points. For instance, 70% of cattle slaughtered in the U.S. are finished within a 200-square-mile area and 80% of these are sent to one of only four packing centers for processing. An attack on one of these "mega-firms" could potentially wipe out a significant portion of the industry (Cupp et al, 2004).

To cause a human disease resulting in a large number of deaths requires some knowledge of the infecting agent as well as an extensive knowledge of microbiology and other related science fields. The technology required can be quite extensive because of the need to maintain viability of the infecting agent, and the difficulty of disseminating the agent so that the infection is ensured. However, an attack on agriculture is quite different. Many plant pathogens are highly contagious to plants and will spread effectively, and only a small number of those pathogens are infectious to humans, reducing risk of infection for the perpetrator. Pathogens that do not infect humans generally are not as strictly monitored or controlled, making them easier to access and handle. These pathogens could be acquired from either a laboratory or the environment. Some

pathogens require only contact with the host to cause an infection, eliminating the need for special weapons or technology that may be required for some human pathogens (Casagrande, 2000). This ease of transmission makes it easy for someone with nefarious intent to cause an infection.

I – d - Biological agents

Biological agents of greatest concern against plants can be grouped into viruses, bacteria, fungi, and nematodes. Viruses consist of a strand or strands of genetic material (DNA or RNA) surrounded by a coat protein. Viruses are dependent on their host, and their effects on plants can be confused with nutrient deficiency, pesticide injury, or other diseases (Strange et al, 2005). Plant viruses differ from other plant pathogens not only because of their submicroscopic size and unique shapes, but also because of their simplified systems of structure, infection, multiplication, dissemination, and chemical composition (Agrios, 2005). Viruses lack the ability to transport themselves; therefore, other dissemination methods are required. The most common is through insects, but some viruses can be disseminated on seeds, in pollen, by fungi, through sap from an infected plant, and through human interaction (Agrios, 2005). Over 2000 different plant viruses are known.

Bacteria are prokaryotic, single-celled organisms, many of which replicate rapidly. Bacteria of various genera infect many plant species and can lead to significant diseases (Strange et al, 2005). The bacterial cell consists of cytoplasm, ribosomes, and DNA that is not bounded by a nuclear membrane. Unlike eukaryotic organisms, bacteria do not contain membrane-bound organelles. Bacteria, the largest group of prokaryotic plant pathogens, can trigger a large variety of host symptoms (Agrios, 2005). Because bacteria lack the ability to penetrate the surface of the host, they must rely on natural openings such as stomata, hydathodes, and wounds, or on insect vectors, to gain access to the internal structure of a plant. Some bacteria form biofilms, sticky

communities of bacteria often found on surfaces of plants. Bacteria also do not have the ability to move long distances (short distances can be achieved via flagella), but are carried by rain, wind, irrigation water, insects, animals, and humans to spread to other plants (Agrios, 2005).

Fungi, the most common plant pathogens, are generally multi-celled, eukaryotic organisms (Strange et al, 2005). Some common forms are yeast, rusts, smuts, mushrooms, and molds. More than 10,000 species of fungi are known to cause disease in plants (Agrios, 2005). Most disease-causing fungi are composed of a filamentous mycelium and branching hyphae. Fungi reproduce by forming spores that germinate, infect, and draw nutrients from the plant. The thick walled nature of the spores allows them to withstand extreme environments. Fungi may spread rapidly by surface water or rain, wind, animals, birds, insects, or humans.

Omycetes are much like fungi because of the mycelial growth and mode of nutrition (Link et al, 2002). However, molecular and morphological studies demonstrate their taxonomic separation. Oomycetes, terrestrial or aquatic organisms, are responsible for many important diseases including downy mildew, white rust, root rot, late blight of potato, and sudden oak death. Germination is species-specific and involves the formation of either germ tubes or zoospores. Sexual reproduction also occurs, providing an optimal mechanism to create genetic variability (Link et al, 2002).

The smallest group of plant pathogens is that of the nematodes; small (300-1000 micrometers long), worm-like animals with unsegmented bodies. Most nematodes live in the soil and feed on the roots of plants, most abundantly in the top 15-30 cm of the soil. The distribution of nematodes in a field is usually irregular, with infection resulting in patches of infected plants within a field. The nematode life cycle is rapid, resulting in rapid spread of infection. Along with their own ability to move, nematodes can be carried by farm equipment, irrigation, floods, and animals (Agrios, 2005). Potato cyst nematodes (PCN) are pathogens of high biosecurity and

quarantine concern (Hodda and Cook, 2009). The nematodes can easily spread to new locations through importation of infected plants, a major concern for the many worldwide locations that are free of the pathogen. PCN has been recently detected in the U.S., Canada, Ukraine, Indonesia, and Australia (Davis et al, 2007; Franko et al, 1998; Marshall, 1998). The significant yield reductions that occur during high infestations make it an important pathogen to control and triggers an emergency response upon initial detection of the pathogen in a new location.

A formal list of organisms and toxins regulated by the U.S. Department of Health and Human Services (HHS) and the U.S. Department of Agriculture (USDA) is called the select agent list (Miller, 2006). The plant pathogen select agent list is composed only of pathogens exotic to the U.S. Once a select agent has arrived and become established in the U.S. it is removed from the list.

The select agent list helps to define what organisms and toxins are considered the most dangerous or threatening, and provides a mechanism of regulating the usage and release of information (Atlas, 2005; Miller, 2006). However, the extensive regulatory processes required to gain permission to work with a listed agent has terminated or hampered the work of many research laboratories, which in some cases may have been ongoing for years before the list was created (Atlas, 2005). Some scientists believe that even the creation of a list can be classified as a dual-use activity because terrorists or criminals gain insight into the agents of interest (Kelley, 2006).

I – e - Prevention and control

Considerable efforts are in place to prevent attacks on our agricultural systems. Vulnerability is being evaluated, and prevention and preparedness strategies are being developed (Wheelis et al, 2002). On June 12, 2002, the U.S. Congress signed into law the Public Health Security and Bioterrorism Preparedness and Response Act. The Act requires the FDA to develop

and implement regulations on the registration of food facilities, prior notice of imported foods, the establishment and maintenance of records, and administrative detention (arrest and detention without trial of individuals that are likely to pose a threat). The regulations created by this act were designed to prepare the U.S. for bioterrorism (Goodrich et al, 2005).

Speed of detection and an effective response plan can significantly reduce the impact of a biological attack (Goodrich et al, 2005; Wheelis et al, 2002). The anthrax letters of 2001 in the U.S. demonstrated the need for a network of laboratories and agencies to efficiently and accurately respond to such events. The ability to trace food products to handlers and growers could decrease response time; this issue is addressed by the Bioterrorism Act of 2002 (Goodrich et al, 2005).

Just as new vaccines are needed to minimize spread of a human or animal disease, new fungicides and pesticides should are needed to reduce the impact of emerging plant diseases (Wheelis et al, 2002). By continuing to study these diseases and pathogens, we will be better prepared for rapid and effective responses.

II - Forensic Science

Forensic science is "using science as a witness to solve crime," as stated by Bruce Budowle, then a scientist with the Federal Bureau of Investigation (FBI), at the 2007 American Phytopathological Society annual meeting. Forensic science has been called a public science (Nordby, 2005) because it combines the interest of the public and criminal attribution with scientific skills. The goal of a forensic scientist is to perform an unbiased analysis of evidence for criminal or civil investigations (Lucas, 1989; Nordby, 2005; Pollack, 1973; Starrs, 1991). The resulting interpretation may come from statistical evidence, such as the percentage of certainty that an unknown DNA sample matches a known DNA sample, or from scientific explanation, as

when a medical diagnosis can account for abnormalities observed (Evett, 1983; Nordby, 2005). In the end, these results and conclusions must withstand the standards of the court of law.

II – a - Forensic procedure

Proper management and investigation of a crime scene is vital to the outcome of a case (Becker and Dale, 2003; Miller, 2005). All evidence must be properly handled (Weston, 1998). Those responsible for investigating and protecting the crime scene may be police officers, crime scene investigators, detectives, or scientific specialists (Weston, 1998). To assure proper examination of the crime scene, a specific methodical process, including physical evidence recognition, documentation, proper collection, packaging, preservation, and scene reconstruction, is followed (Adams and Krutsinger, 2000; Miller, 2005).

Crime scene investigation is only as good as the management supporting the investigation. Important to the success of the investigation are information management, manpower management, technology management, and logistics management (Becker and Dale, 2003; Miller, 2005). Managers provide communication and coordination throughout the entire investigative process.

Police officers, fire department personnel, and emergency medical personnel, often the first responders, should assist any victims, search for and arrest suspect(s), detain witnesses, protect the crime scene, and communicate to the crime scene investigators (Miller, 2005). Safety is always the first concern, but thorough documentation of the scene is also vital (Jamieson, 2004; Miller, 2005; Scott, 2009a; Trestrail, 2007). The first responder must secure and restrict access to the scene, as any movement in the area has the potential of altering or changing the evidence (Miller, 2005).

Once the crime scene investigator arrives at the scene, a preliminary walk-through is performed to help reconstruct the scene, identify evidence needing immediate attention, prepare

for weather or unanticipated conditions, identify entry/exit points and paths taken by first responders, record initial observations, and evaluate the need for specialists or special equipment (Jamieson, 2004; Miller, 2005; Scott, 2009a; Trestrail, 2007).

The most important step in crime scene investigation is documentation (Jamieson, 2004; Miller, 2005; Scott, 2009a; Trestrail, 2007). After the preliminary walk-through, the crime scene investigator should document everything they encountered. This is time-consuming and requires accurate organization. The four required types of documentation are note taking, videography, photography, and sketching (Miller, 2005; Scott, 2009b). When possible, the notes should include who, what, when, why, where, and how. Information related to notification, arrival, scene description, victim description, and crime scene team also should be included (Scott, 2009b). Video taping can be helpful because it provides a three dimensional aspect. The tape should document case information, scene surroundings, orientation of the scene, and victims' viewpoint. Multiple camera techniques may be used, including varying lighting (Miller, 2005; Scott, 2009b). Just like videography, photography records the initial condition of the scene, but it also includes pictorial documentation of specific evidence (Grip et al, 2000; Miller, 2005). Photos should always be taken starting with a general picture and working towards more specific, close-up photos. Finally, sketching allows for documentation supplemented with measurements and units, which allows for the correct perspective and placement of physical evidence (Miller, 2005; Scott, 2009b). Two types of sketching are used, a rough sketch and a finished or final sketch, both of which are drawn as both an overhead view and a side view. Measurements can be made using triangulation, a base-line, or polar coordinates; all three are based on two fixed points such as building corners, utility poles, or trees.

To search a crime scene for physical evidence, search methods typically involve geometric patterns such as link, line and strip, grid, zone, wheel or ray, and spiral (Bates and Lambert, 1991). Each pattern has disadvantages and advantages and is appropriate for a

particular type of scene (Miller, 2005). Once physical evidence is found, it must be properly collected and preserved. An evidence collector is identified to ensure consistent collecting and packaging methods (Miller, 2005). Most evidence is collected in a primary container and packaged again in a secondary container. The outer container is sealed with tamper-resistant tape and marked with the initials of the collector over the tape. The container should also be marked with information about the evidence. To prevent contamination, each item is packaged separately in the proper container, with the container selected based on the type of evidence. For example, liquid or volatile evidence is packaged in an airtight, unbreakable container, while most biological evidence is packaged temporarily in a non-airtight container (Budowle et al, 2006; Miller, 2005).

The final step in the basic crime scene investigation is reconstruction. This includes determining both what happened and what did not happen at the scene. Reconstruction is based on the appearance, location, and position of physical evidence, and laboratory examination of that evidence (Jamieson, 2004; Miller, 2005; Scott, 2009a; Trestrail, 2007). The method of reconstruction follows the general scientific method: data is collected, an initial idea is formed, more data accumulation leads to a hypothesis, testing is done to prove or disprove the hypothesis, and a theory is formed.

Several scientific specialties, such as pathology, toxicology, odontology, and anthropology, exist within the science of forensics, allowing for in depth and accurate examination of the crime scene and evidence collected. The pathologist performs autopsies and identifies the time, cause, and course of death (Wright, 2005). The toxicologist analyzes bodily fluids for presence of toxins or poisons, and also determines the cause of death (Fenton, 2005; Anderson, 1998). Odontologists and anthropologists determine the identification or physical characterization of the victims, but odontologists analyze teeth while anthropologists analyze the human remains, most commonly bones (Glass, 2005; Sorg, 2005).

II – b - Laboratory techniques

DNA, blood, semen, hair, fibers, glass, and paint are examples of physical evidence used to piece together the events of a crime and ultimately lead to attribution (Netzel, 2005). As stated by Paul Kirk, "physical evidence cannot be wrong…only in its interpretation can there be error" (Netzel, 2005). Laboratory capabilities are constantly growing because of the enhanced technology. Old cases are even being revisited because of advances in technology, such as DNA analysis (Miller, 2005; Neufeld, 2000).

Biological evidence, the domain of a forensic biologist, traditionally includes hair, blood, semen, saliva, and many other tissues (Netzel, 2005), but also includes other biological samples, such as plant tissue, seeds, arthropods, and microbes from soil, water, and plants, which may be encountered during an agroterrorism investigation (Budowle et al, 2006; Fletcher et al, 2006). Serology was used to process most of this evidence in the past, but now DNA analysis is the primary tool used (Jobling and Gill, 2004; Netzel, 2005; Smith and Gordon, 1996). An early method of DNA analysis was restriction fragment length polymorphism (RFLP). In this method, restriction enzymes are used to cut the extracted DNA into fragments, followed by separation of the fragments by size by gel electrophoresis. The DNA fragments are then transferred to a membrane, hybridized to a labeled probe, and exposed on film, producing a lumigraph. The quantity and quality of the DNA needed to produce useable results was a disadvantage (Budowle et al, 1995; Netzel, 2005).

Polymerase chain reaction (PCR) is more promising for analyzing genetic material. PCR amplifies DNA *in vitro* by mimicing DNA replication performed *in vivo*. The advantage of PCR is the ability to use small quantities and/or partially degraded DNA (Budowle et al, 1995, 2005c; Della Manna and Montpetit, 2000; Netzel, 2005). PCR is now the technique most commonly used for DNA analysis in forensic laboratories and also plant disease diagnostic laboratories.

Several types of genetic markers can be utilized in PCR to create a genetic profile of an individual or sample, the most common for human forensic testing being short tandem repeats (STR). The profile created by the number of STRs in a set number of loci on autosomal chromosomes (and occasionally Y-chromosomes) is utilized in parentage testing (Budowle et al, 1991, 2001; Coble et al, 2006; Hochmeister, 1995; Jobling et al, 1997). A similar process is used for maternal determination using mitochondrial DNA (Hochmeister, 1995; Wilson et al, 1993). By querying numerous loci in the procedure and comparing the profile to a database containing thousands of profiles, a high level of confidence can be achieved for determining the likelihood the unknown sample matches the reference sample (Budowle et al, 2001, 2005a; Netzel, 2005).

Many of these molecular typing techniques are also common techniques used for genetic discrimination of microorganisms and could be used to compare biological evidence of this type. For example, using amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and pulse field gel electrophoresis (PFGE), 44 genotypes of *Pseudomonas aeruginosa* were identified from hospital samples, which lead to the determination of the origin of this bacterium (Speijer, 1999). AFLP was also utilized to determine the phylogenetic relationships of taxa within the genus *Bacillus*, but was unsuccessful at discriminating among *Bacillus anthracis* genotypes because of the low level of diversity (Keim et al, 1997). Genotypic variants of *Fusarium graminearum* and *F. colmorum* containing a particular virulence factor were detected using RAPD, leading to a better understanding of epidemiological factors influencing these wheat pathogens (Nicholson et al, 1998). Additionally, genotype characterization was performed on the rice blast fungus, *Magnaporthe grisea*, using microsatellites, also known as simple sequence repeats (SSR), providing an understanding of pathogenicity of the population (Kim et al, 2000) . Rapid detection and strain discrimination for plant pathogens at ports of entry is important to reduce introduction of exotic or quarantined pathogens. PCR is an excellent tool to use in this setting, such as the one-hour real-time PCR

method developed for the identification of the Pierce's Disease strain of *Xylella fastidiosa* (Shaad et al, 2002). These are only a few examples of the wide array of molecular typing techniques available (Mitchelson and Morrica, 2008; Schaad et al, 2003)

Another method that is being explored for genetic analysis uses single nucleotide polymorphisms (SNPs). SNPs occur when a nucleotide(s) is(are) different between members of the same species. These polymorphisms may or may not result in a phenotypic change, such as genetic disorders, depending on the location of the SNP. Many techniques are available for SNP analysis, with most requiring hybridization of the template to a target-specific oligonucleotide, but using different detection platforms. Affymetrix and Illumina SNP arrays have been developed to detect and characterize over 10 million known SNPs in the human genome on a single microarray (Int'l SNP Map Working Group, 2001; LaFramboise, 2009; Wang et al, 1998). Following hybridization of allele-specific oligonucleotides, detection of the SNPs is achieved through measuring the signal intensities of each reaction (LaFramboise, 2009; Wang et al, 1998). A similar technique involving SNP-specific oligonucleotide hybridization to the template has been developed, but instead of using a microarray, this method utilizes single base extension PCR to incorporate a fluorescently-labeled nucleoside into the position complementary to the polymorphic site. The ABI PRISM SNaPshot multiplex kit is one kit that utilizes this technology and has been used to characterize organisms into serogroups or pathotypes, such as *Eschercholia coli* and *Potato Virus* Y (Hommais et al, 2005; Rolland et al, 2008), to identify mutations responsible for diseases in animals (Bujakova et al, 2008; Murphy et al, 2007), and for detection of desirable traits in plants and animals (Ceriotti et al, 2004; Civaova and Knoll, 2007; Kawuki et al, 2009; Ovesna et al, 2003; Van et al, 2005). A colorometric detection technique has also been developed using specific oligonucleotides and DNA ligase (Li et al, 2005). The reactions containing mismatches between the target and oligonucleotide cannot undergo ligation, resulting in a color change as compared to the reactions containing a perfect match.

Using SNPs to generate a genetic profile can be advantageous over other genotyping methods because the profile can be generated from a template of poor quality (Norton et al, 2002; Quintans et al, 2004). By using multiple SNPs and targeting specific sites within the genome, more information can be achieved than other methods that are gel based, such as RFLP or MLVA, without requiring a more extensive procedure (Kohnemann et al, 2008). However, to generate a SNP profile using most techniques, oligonucleotides specific to each target must be designed which can be a limitation if the sequence of the organism is unavailable (Budowle et al, 2005c, 2010).

Laboratory techniques for non-biological samples use microscopy to compare known and unknown samples, such as fibers, bullets, cartridge casings, glass, tool mark impressions, rocks, and soil (Ruffell and McKinley, 2005). Depending on the type of evidence and the extent of detail needed, the appropriate tool may be a polarizing light microscope, phase contrasting light microscope, comparison microscope, or a scanning electron microscope (Taylor, 1973). Evidence relating to chemical make-up of a material such as paint may be analyzed using a Fourier transform infra-red spectrometer, providing a "chemical fingerprint" (de Chazal et al,2005; Netzel, 2005; Watling et al, 1997).

II – c - Microbial forensics

A developing area of forensic science, microbial forensics is defined as "a scientific discipline dedicated to analyzing evidence from a bioterrorism act, biocrime, or inadvertent microorganism/toxin release for attribution purposes" (Budowle et al, 2003). As the threat of microorganisms as weapons has become more apparent, multiple national groups are working to increase capability in microbial forensics (Budowle et al, 2003, 2005a). In 2002, the FBI established the Scientific Working Group on Microbial Genomics and Forensics (SWGMGF). More recently, the Department of Homeland Security (DHS) established the National Biodefense

Analysis and Countermeasures Center (NBACC), bringing together public health, veterinary science, plant pathology, law enforcement, and national security elements to create a national focus on homeland security. For a microbial forensics investigation involving plant diseases, the already developed network of plant disease diagnostic laboratories in the U.S., known as the National Plant Diagnostic Network (NPDN), can be a vital resource. The network provides an avenue for information exchange and detection and diagnostic capabilities are already set up (Bostock et al, 2007; Stack et al, 2006, 2007)

Identifying the perpetrator(s) of a biological crime is important, but it is also important to identify a pathogen's origin and when and how it was introduced (Fletcher et al, 2006). Microbial forensic investigations should follow the same process used in other forensic areas: crime scene investigation, maintenance of chain of custody, collection of evidence, handling and preservation, shipping of evidence, analysis, interpretation of results, and presentation of the evidence in court (Budowle et al, 2005a, 2005b). Since this is a new field, the details of the investigative process are still being developed, and gaps exist in key areas (Budowle et al, 2003).

Methods of plant disease diagnosis and detection are already available, but the stringent demands of a criminal investigation and the pressures of the courtroom require that these methods be revised and validated for forensic use (Budowle et al, 2005a, 2005b, 2005c, 2005d; Fletcher et al, 2006). Other aspects of forensic investigation that must be optimized for plant disease applications include sampling, packaging, shipping, storage, identification, and discrimination (Fletcher et al, 2006).

High priorities include detection and identification assays, readily accessible databases of genomic sequences and other biological data, and microbial strain repositories containing representative pathogen strains (Murch, 2003; Tucker and Koblentz, 2009). Methods and quality assurance (QA) guidelines must be validated prior to an event (Budowle et al, 2005a).

Another area of concern with respect to forensic plant pathology is the scarcity of personnel trained in both applied plant pathology and forensic science. The U.S. Cooperative Extension Service has served as a link between diagnostic laboratories, land-grant universities, and producers for many years, but recently has been understaffed, in part because of reduced funding and in part because of a shift in graduate student research to fundamental instead of applied science. Targeted funds are needed for education and training in applied plant pathology (Fletcher et al, 2006).

Bioforensic efforts are limited also by the lack of high-level containment (BSL-3 and BSL-4) facilities required for handling high-risk pathogens, which are expensive to build and often incite resistance from local citizenry concerned about the potential for inadvertent escapes. New containment labs can be constructed in locations where an accidental release of a high-risk pathogen would cause no consequences, such as a geographical location where no plant hosts are available (Fletcher et al, 2006).

Finally, forensic plant pathology is limited by a lack of research and technology development. Rapid and efficient protocols are needed to achieve attribution, and methods need to be adapted for specific crime scenes, such as crops, forests, nurseries, orchards, or rangelands (Fletcher et al, 2006).

III - *Wheat Streak Mosaic Virus*

Wheat streak mosaic virus (WSMV), one of the most important pathogens of wheat in the U.S., is composed of a positive sense, single-stranded RNA genome encapsidated by capsid proteins into a long, flexuous virion, approximately 700 nm in length (Niblett et al, 1991). WSMV belongs to the genus *Tritimovirus* within the family *Potyviridae* (Stenger et al, 2002). The virus is 9384 nucleotides (nt) long and is translated into a single polyprotein, which is

processed by three viral proteinases (Stenger et al, 2002). The 1213nt coat protein coding region is located near the 3' end of the genome (Niblett et al, 1991).

WSMV is common in many wheat-growing regions, including North America, Europe, the Middle East, and most recently Australia (Bowen et al, 2003; Burges et al, 2003; Dwyer et al, 2007; Kapooria and Ndunguru, 2004; Murray et al, 2005; Sanchez-Sanchez et al, 2001; Signoret, 1974). Its name comes from the primary host, wheat, and the predominant disease symptom, the mosaic pattern formed on the leaves. Light green streaks parallel to the leaf veins later turn yellow, creating green and yellow blotches (McKinney, 1949). Stunting, sterile wheat heads, and premature death are other common symptoms (Stromberg, 2003).

While wheat is the preferred host of WSMV, other cereals and grasses including barley, oat, rye, maize, brome, blackgrass, crowsfoot, and couch grass are also prone to infections (Christian and Willis, 1993; McKinney, 1949; Sill and Connin, 1953). WSM symptoms and damage are most obvious on wheat; other hosts typically show few, if any, symptoms.

WSMV, like most plant viruses, is unable to spread from plant to plant on its own. The only known vector of WSMV is the eriophyid wheat curl mite (*Aceria tosichella* Keifer; Connin, 1956; Keifer, 1936; Somsen and Sill, 1970; Slykhuis, 1955), which is 0.2 mm long, white to yellow, and cigar-shaped, with two pairs of legs at the front of the body. Most, eriophyid mite species are host-specific, but wheat curl mites feed on a large number of cereals and grasses (Somsen and Sill, 1970). The mite, because of its microscopic size, is not a quick mover, but relies on wind or insects to take it to other plants near and far (Somsen and Sill, 1970.).

WSMV, picked up by mites feeding on an infected plant, survives in the mite for life (~10 days total), and is passed on to healthy plants during feeding. The virus is retained through molting, but cannot be passed on to the next generation of mites (Somsen and Sill, 1970). Mite and viral spread are influenced by environmental conditions, mainly temperature, moisture, and

wind speed. Most mite movement occurs at the end of the spring when winter crops and grasses have matured, and at the end of the summer when oversummering grasses have matured. Ideal conditions for mite movement are a temperature of 18°C and a wind speed of 25 km/h or greater (Murray et al, 2005). A moist summer promotes growth of volunteer wheat, which serves as an oversummering host for the mite. This allows the virus to remain viable for the following year (Connin, 1959; Slykhuis, 1955; Somsen and Sill, 1970; Thomas and Hein, 2003).

Control of Wheat streak mosaic (WSM), the disease caused by WSMV, is primarily achieved by the removal of oversummering hosts for the wheat curl mite; these hosts are known as a "green bridge" because they provide a living host between harvest and fall emergence (Bowden et al, 1991). Destroying the volunteer wheat in the field and surrounding areas by cutting of the plants, applying glyphosate herbicides, or withholding water several weeks before planting is an effective way to manage insects, mites, and virus (Hesler et al, 2005; Jiang et al, 2005; Somsen and Sill, 1970). Delaying planting date is also an effective method of control as the delay helps to avoid the primary time of insect and mite movement (Hesler et al, 2005; Hunger et al, 1992; Somsen and Sill, 1970). A few WSM resistant wheat varieties are available, but these have a significantly lower yield potential than other varieties, and are generally used only when WSM is consistently present at a high severity every year (Baley et al, 2001; Somsen and Sill, 1970).

Like many RNA viruses, WSMV is known to exist as a quasi-species, in which multiple variants of the virus persist in a single population (French and Stenger, 2005). The primary source of the variation is believed to be the error-prone RNA-dependent RNA polymerase, whose lack of a proofreading function results in frequent genomic sequence errors (French et al, 2005). WSMV variants have been characterized by several groups (Stenger et al, 2002; French et al, 2003, 2005; Hall et al, 2001a, 2001b; Choi et al, 2001; Rabenstein et al, 2002). Studies of diversity among strains from different geographical locations revealed high pairwise sequence

identities and evidence of both low genetic diversity and high genetic stability (Stenger et al, 2002; Rabenstein et al, 2002). Even though the strains are genetically similar (Figure 3), a substantial number of polymorphic sites exist (Stenger et al, 2002). WSM was first detected in the U.S. in the early 1920s (McKinney, 1937). The accumulation of the virus in the U.S. over nearly 100 years has allowed a widespread distribution of the virus. The U.S. population is suspected to be near genetic saturation, meaning the level of diversity found within a specific field or region is equivalent to that found across the nation (Choi et al, 2001; French and Stenger, 2003; Hall et al, 2001a, 2001b).

Genetic analysis of the WSMV genome has revealed many conserved regions as well as a few genes that are highly variable, including the coat protein (CP), protein 1(P1), protein 3 (P3), and regions of the helper component-protease (HC-Pro) (Choi et al, 2001; Stenger et al, 2002, French and Stenger, 2005; Shukla et al, 1991). A 1371 nt region encompassing the CP and the flanking regions contained 189 polymorphic sites of which 158 sites were singletons (a specific mutation appearing only once in a single clone throughout the entire dataset) and the remaining 31 were shared mutations (identical mutations appearing at the same site in multiple clones) (French et al, 2005). This diversity of the CP region may be a result of the adaptations needed to survive different environments (Choi et al, 2001; French and Stenger, 2003, 2005; Hall et al, 2001a, 2001b; Stenger et al, 2002). P1 is suspected to be a suppressor of silencing and also involved in proteolytic cleavage while P3 is one of the proteins responsible for movement and replication (Choi et al, 2002, 2005; Stenger et al, 2007). HC-Pro is involved in eriophyid mite transmission and viral protein cleavage (Stenger and French, 2004; Stenger et al, 2005a, 2005b, 2006a, 2006b; Young et al, 2007). Molecular studies of the HC-Pro protein have identified specific amino acids that are pertinent for each function and several conserved domains among potyviruses and tritimoviruses (Stenger and French, 2004; Stenger et al, 2005b, 2006a, 2006b).

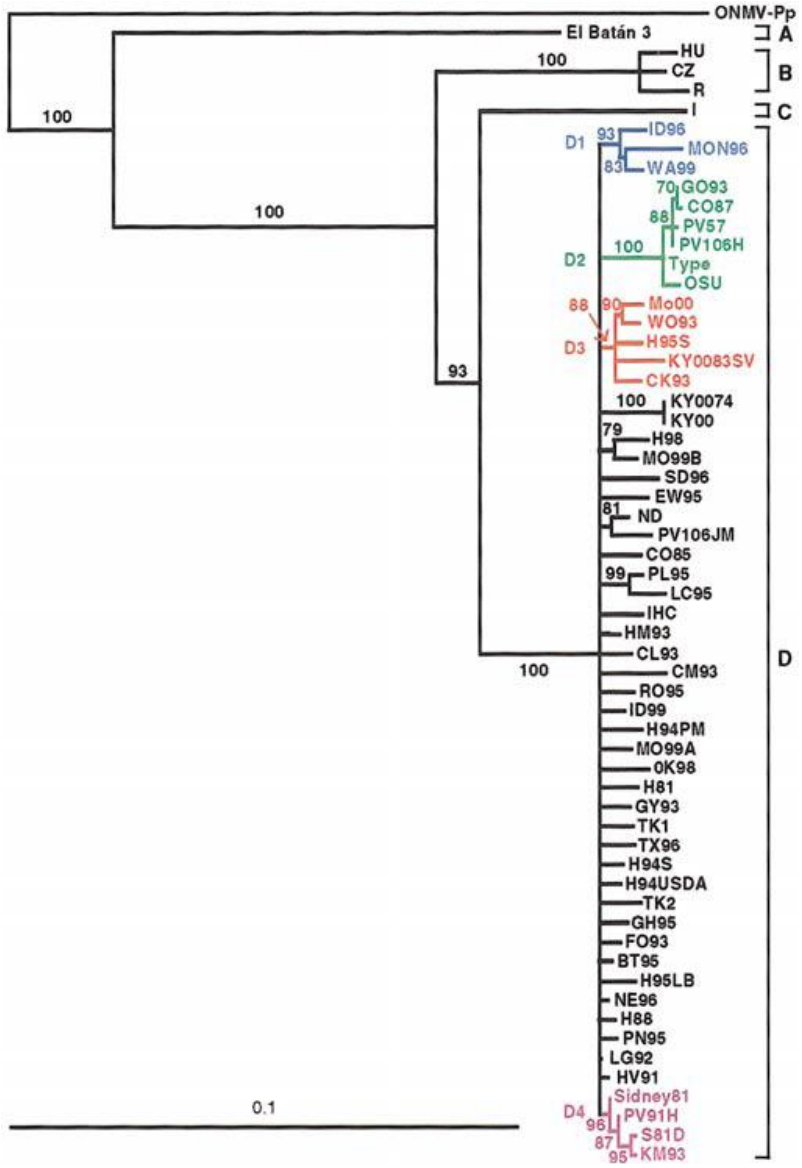


Figure 3: Phylogram of U.S. strains of WSMV. (Figure 1 in Stenger et al, 2002)

LITERATURE CITED

Adams T.F., and Krutsinger J.L. 2000. Crime Scene Investigation. Prentice Hall Publishing, Upper Saddle River, NJ.

Agrios, G. N. 2005. Plant Pathology, 5th edition. Elsevier Academic Press. London, UK.

Anderson, R. 1998. Chapter 10: Forensic toxicology. p. 232-262. *In* P. White (ed.), Crime scene to court: the essentials of forensic science. The Royal Society of Chemistry, Cambridge, UK.

Atlas, R. M. 2005. Biosecurity concerns: Changing the face of academic research. Chemical Health and Safety, 12:15-23.

Baley, G. J., Talbert, L. E., Martin, J. M., Young, M. J., Habernicht, D. K., Kushnak, G. D., Berg, J.E., Lanning, S.P., and Bruckner, P. L. 2001. Agronomic and end-use qualities of *Wheat streak mosaic virus* resistant spring wheat. Crop Sci. 41:1779-1784.

Bates J.W., and Lambert J.A. 1991. Use of the hypergeometric distribution for sampling in forensic glass comparison. Journal of Forensic Science Society 31:449-455.

Becker W.S., and Dale W.M. 2003. Strategic human resource management in the forensic science laboratory. Forensic Science Communications. 5.

Bostock R., Thomas C., Hoenisch R., and Coggeshall A. 2007. The National Plant Diagnostic Network - role in agricultural biosecurity. Phytopathology 97:S166.

Bowden, R. L., Brooks, H. L., Peterson, D. E., and Shroyer, J. P. 1991. Be a good neighbor: control your volunteer wheat. Kansas State University Agricultural Experiement Station Cooperative Extension Services, MF-1004.

Bowden, R. L. 2000. *Wheat streak mosaic virus*. Kansas State University. http://www.plantpath.ksu.edu/DesktopDefault.aspx?tabid=528

Bowen, K. L., Murphy, J. F., Flanders, K. L., Mask, P. L., and Li, R. 2003. Incidence of viruses infecting winter wheat in Alabama. Plant Dis. 87:288-293.

Bronze, S. B., and R. A. Greenfield. 2005. Introduction, p. 1-23. *In* M.S. Bronze, R. S. Greenfield (ed.), Biodefense: Principles and Pathogens. Horizon Bioscience, Norfolk, England.

Budowle B., Giusti A.M., Waye J.S., Baechtel F.S., Fourney R.M., Adams D.E., Presley L.A., Deadman H.A., and Monson K.L. 1991. Fixed-bin analysis for statistical evaluation of continuous distributions of allelic data for VNTR loci, for use in forensic comparisons. Am. J. Hum. Genet. 48:841-855.

Budowle B., Baechtel F.S., Comey C.T., Giusti A.M., and Klevan L. 1995. Simple protocols for typing forensic biological evidence: Chemiluminescent detection for human DNA quantitation and restriction fragment length polymorphism (RFLP) analyses and manual typing of polymerase chain reaction (PCR) amplified polymorphisms. Electrophoresis 16:1559-1567.

Budowle, B., Shea, B., Niezgoda, S., and Chakraborty, R. 2001. CODIS STR loci data from 41 sample populations. J Forensic Sci. 46:453-489.

Budowle, B., S. E. Schutzer, A. Einseln, L. C. Kelley, A. C. Walsh, J. A. Smith, B. L. Marrone, J. Robertson, and J. Campos. 2003. Public health. Building microbial forensics as a response to bioterrorism. Science 301: 1852-1853

Budowle, B., J. Burans, R. G. Breeze, M. R. Wilson, and R. Chakraborty. 2005a. Microbial forensics, p. 1-26. *In* R. G. Breeze, B. Budowle, and S. E. Schutzer (ed.), Microbial Forensics. Elsevier Academic Press, San Diego, Calif.

Budowle, B., R. S. Murch, and R. Chakraborty. 2005b Microbial forensics: the next forensic challenge. Int. J. Leg. Med. 119: 317-330

Budowle, B., Johnson, M. D., Fraser, C. M., Leighton, T. J., Murch, R. S., and Chakraborty, R. 2005c. Genetic analysis and attribution of microbial forensics evidence. Crit. Rev. Microbiol. 31:233-254.

Budowle, B., Schutzer, S. E., Ascher, M. S., Atlas, R. M., Burans, J. P., Chakraborty, R., Dunn, J. J., Fraser, C.M., Franz, D.R., Leighton, T.J., Morse, S.A., Murch, R., Ravel, J., Rock, D.L., Slezak, T.R., Velsko, S.P., Walsh, A.C., and Walters, R. A. 2005. Toward a system of Microbial Forensics: from sample collections to interpretation of evidence. Appl. Environ. Microb. 71:2209-2213.

Budowle B., Schutzer S.E., Burans J.P., Beecher D.J., Cebula T.A., Chakraborty R., Cobb W.T., Fletcher J., Hale M.L., Harris R.B., Heitkamp M.A., Keller F.P., Kuske C., LeClerc J.E., Marrone B.L., McKenna T.S., Morse S.A., Rodriquez L.L., Valentine N.B., and Yadev J. 2006. Quality sample collection, handling, and preservation for an effective microbial forensics program. Appl. Environ. Microb. 72:6431-6438.

Burges, N., R. Jones, and S. Kumar. 2003. *Wheat streak mosaic virus*. Government of Western Australia, Department of Agriculture factsheet, ISSN: 1448-0344

Bujalkova M., Zavodna K., Krivulcik T., Ilencikova D., Wolf B., Kovac M., karner-Hanusch J., Heinimann K., Marra G., jiricny J., and Bartosova Z. 2008. Multiplex SNaPshot

genotyping for detecting loss of heterozygosity in the mismatch-repair genes *MLH1* and *MSH2* in microsatellite-unstable tumors. Clin. Chem. 54:1844-1854.

Cameron, G., and J. Pate. 2001. Covert biological weapons attacks against agricultural targets: assessing the impact against US agriculture. Terror. Polit. Violenc. 13:61-82.

Carver, J. D. 2007. Molecular typing of *Wheat streak mosaic virus* for forensic applications. M.S. thesis, Oklahoma State University Center for Health Sciences.

Casagrande, R. 2000. Biological terrorism targeted at agriculture: the threat to U.S. national security. Nonproliferation Rev. 73:92-105.

Ceriotti G., Chessa S., Bolla P., Budelli E., Bianchi L., Duranti E., and Caroli A. 2004. Single nucleotide polymorphisms in the ovine casein genes detected by polymerase chain reaction-single stranded conformation polymorphism. J. Dairy Sci. 87:2606-2613.

Chenault, K. D., Hunger, R. M., and Sherwood, J. L. 1996. Comparison of the nucleotide sequences of the coat protein open reading frame of nine isolates of *Wheat streak mosaic rymovirus*. Virus Genes 13:187-188.

Choi, I. R., J. S. Hall, M. Henry, L. Zhang, G. L. Hein, R. French, and D. C. Stenger. 2001. Contributions of genetic drift and negative selection on the evolution of three strains of *Wheat streak mosaic tritimovirus*. Arch. Virol. 146: 619-628

Choi, I.-R., Horken, K. M., Stenger, D. C., and French, R. 2002. Mapping of the P1 proteinase cleavage site in the polyprotein of *Wheat streak mosaic virus* (genus *Tritimovirus*). J. Gen. Virol. 83:443-450.

Choi, I.-R., Horken, K. M., Stenger, D. C., and French, R. 2005. An internal RNA element in the P3 cistron of *Wheat streak mosaic virus* revealed by synonymous mutations that affect both movement and replication. J. Gen. Virol. 86:2605-2614.

Christian, M. L., and Willis, W. G. 1993. Survival of *Wheat streak mosaic virus* in grass hosts in Kansas from wheat harvest to fall wheat emergence. Plant Dis. 77:239-242.

Civaova K., and Knoll A. 2007. SNaPshot minisequencing and a panel of candidate genes for complex routine testing of meat performance traits in pigs. Anim. Biotechnol. 18:109-115.

Coble M.D., Hill C.R., Vallone P.M., and Butler J.M. 2006. Characterization and performance of new miniSTR loci for typing degraded samples. Int. Congr. Ser. 1288:504-506.

Connin, R. V. 1956a. The host range of the wheat curl mite, vector of wheat streak-mosaic. J. Econ. Entomol. 49:1-4.

Connin, R. V. 1956b. Oversummering volunteer wheat in the epidemiology of wheat streak mosaic. J. Econ. Entomol. 49:405-406.

Cupp, O. S., D. E. Walker II, and J. Hillison. 2004. Agroterrorism in the U.S.: key security challenge for the 21st Century. Biosecur. Bioterror. 2: 97-105.

Davis E., McNeal D., El-Lissy O., Berger P., and Bulluck R. 2007. Current situation of potato cyst nematodes in Idaho. Phytopathology 97:S151.

de Chazal P., Flynn J., and Reilly R.B. 2005. Automated processing of shoeprint images based on the Fourier transform for use in forensic science. IEEE T. Pattern Anal. 27:341-350.

Della Manna A., and Montpetit S. 2000. A novel approach to obtaining reliable PCR results from lumino treated bloodstains. J. Forensic Sci. 45:886-890.

Duval, J. S. B, C. M. Dougherty, and M. K. Sullivan. 2005 Chapter 3: Forensic nursing. p. 27-42. *In* S. H. James and J. J. Nordby (ed.), Forensic Science: An introduction to scientific investigative techniques. 2nd edition. CRC Press, Boca Raton, Florida

Dwyer, G. I., Gibbs, M. J., Gibbs, A. J., and Jones, R. A. C. 2007. *Wheat streak mosaic virus* in Australia: Relationship to isolates from the Pacific Northwest of the USA and its dispersion via seed transmission. Plant Dis. 91:164-170.

Evett I.W. 1983. What is the Probability that This Blood Came from That Person? A Meaningful Question? Journal of the Forensic Science Society 23:35-39.

Fenton, J. J. 2005. Chapter 5: Forensic toxicology. p. 61-78. *In* S. H. James and J. J. Nordby (ed.), Forensic Science: An introduction to scientific investigative techniques. 2nd edition. CRC Press, Boca Raton, Florida.

Fletcher, J., C. Bender, B. Budowle, W. T. Cobb, S. E. Gold, C. A. Ishimaru, D. Luster, U. Melcher, R. Murch, H. Scherm, R. C. Seem, J. L. Sherwood, B. W. Sobral, and S. A. Tolin. 2006. Plant pathogen forensics: capabilities, needs, and recommendations. Microbiol. Mol. Biol. Rev. 70: 450-471.

Fletcher, J., and U. Melcher. 2006a. Microbes in Crime: Decision trees for interpreting evidence. Unpublished.

Franco J., Oros R., Main G., and Ortuno N. 1998. Potato cysts nematodes (*Globodera* species) in South America, in: R. J. Marks and B. B. Brodie (Eds.), Potato cyst nematodes, biology, distribution and control, CAB International, Wallingfor, UK. pp. 239-269.

Fraser, E. D. G. 2003. Social vulnerability and ecological fragility: building bridges between social and natural sciences using the Irish Potato Famine as a case study. Conserv. Ecol. 7: 9.

French, R., and D. C. Stenger. 2003. Evolution of *Wheat streak mosaic virus*: Dynamics of population growth within plant may explain limited variation. Annu. Rev. Phytopathol. 41: 199-214.

French, R., and D. C. Stenger. 2005. Population structure within lineages of *Wheat streak mosaic virus* derived from a common founding event exhibits stochastic variation inconsistent with the deterministic quasi-species model. Virology. 343: 179-189.

Fry, E. W., and S. B. Goodwin. 1997. Resurgence of the Irish Potato Famine fungus. BioScience. 47: 363-371.

Gallop, A. and R. Stockdale. 1998. Chapter 3: Trace and contact evidence. p. 47-72. *In* P. White (ed.), Crime scene to court: the essentials of forensic science. The Royal Society of Chemistry, Cambridge, UK.

Ge, J. Y., Budowle, B., Planz, J. V., and Chakraborty, R. 2010. Haplotype block: a new type of forensic DNA markers. Int. J. Legal Med. 124:353-361.

Glass, R. T. 2005. Chapter 6: Forensic ondontology. p. 79-98. *In* S. H. James and J. J. Nordby (ed.), Forensic Science: An introduction to scientific investigative techniques. 2nd edition. CRC Press, Boca Raton, Florida.

Goodrich, R. M., K. R. Schnieder, C. D. Webb, and D. L. Archer. 2005. Agroterrorism in the U.S.: an overview. University of Florida, Gainsville. Food Safety and Biosecurity White Paper Series: FSHN05-21.

Greene, J. S., and E. Maxwell. 2007. Climatic impacts on winter wheat in Oklahoma and potential applications to climatic and crop yield prediction. Int. J. Biometeorol. 52: 117-126.

Grip W.M., Grip R.W., and Morrison R.D. 2000. Application of aerial photography and photogrammetry in environmental forensic investigations. Environ. Forensics 1:121 - 129.

Grunow, R., and E. –J. Finke. 2002. A procedure for differentiating between the intentional release of biological warfare agents and natural outbreaks of disease: its use in analyzing the tularemia outbreak in Kososvo in 1999 and 2000. Clin. Microbiol. Infec. 8: 510-521.

Hall, J. S., R. French, T. J. Morris, and D. C. Stenger. 2001a. Structure and temporal dynamics of populations within *Wheat streak mosaic virus* isolates. J. Virol. 75: 10231-10243.

Hall, J. S., R. French, G. L. Hein, T. J. Morris, and D. C. Stenger. 2001b. Three distinct mechanisms facilitate genetic isolation of sympatric *Wheat streak mosaic virus* lineages. Virology. 282: 230-236.

Hesler, L. S., Riedell, W. E., Langham, M. A., and Osborne, S. L. 2005. Insect infestations, incidence of viral plant diseases, and yield of winter wheat in relation to planting date in the northern great plains. J. Econ. Entomol. 98:2020-2027.

Hickey, D. J., and J. E. Doherty. 1980. Famine of 1845-49. p. 160-164. *In* D. J. Hickey, J. E. Doherty (ed.), A dictionary of irish history since 1800. Gill and MacMillian, Dublin.

Hodda M., and Cook D.C. 2009. Economic impact from unrestricted spread of potato cyst nematodes in Australia. Phytopathology 99:1387-1393.

Hommais F., Pereira S., Acquaviva C., Escobar-Paramo P., and Denamur E. 2005. Single-nucleotide polymorphism phylotyping of *Escherichia coli*. Appl. Environ. Microbiol. 71:4784-4792.

Hunger, R. M., Sherwood, J. L., Evans, C. K., and Montana, J. R. 1992. Effects of planting date and inoculation date on severity of Wheat streak mosaic in hard red winter wheat cultivars. Plant Dis.76:1056-1060.

International SNP Map Working Group. 2001. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. Nature 409:928-933.

James, S. H., P. E. Kish, and T. P. Sutton. 2005. Chapter 11: Recognition of bloodstain patterns. p. 189-218. *In* S. H. James and J. J. Nordby (ed.), Forensic Science: An introduction to scientific investigative techniques. 2nd edition. CRC Press, Boca Raton, Florida.

Jamieson A. 2004. A rational approach to the principles and practice of crime scene investiation: I. Principles. Sci. Justice 44:3-7.

Jiang, W., Garrett, K. A., Peterson, D. E., Harvey, T. L., Bowden, R. L., and Fang, L. 2005. The window of risk for emigration of *Wheat streak mosaic virus* varies with host eradication method. Plant Dis. 89:853-858.

Jobling M.A., Pandya A., and Tyler-Smith C. 1997. The Y chromosome in forensic analysis and paternity testing. Int.J. Legal Med. 110:118-124.

Jobling M.A., and Gill P. 2004. Encoded evidence: DNA in forensic analysis. Nat. Rev. Genet. 5:739-751.

Kapooria, R. G., and Ndunguru, J. 2004. Occurence of viruses in irrigated wheat in Zambia. Bulletin OEPP/EPPO Bulletin, 34:413-419.

Kawuki R.S., Ferguson M., Labuschagne M., Herselman L., and Kim D.-J. 2009. Identification, characterization and application of single nucleotide polymorphisms for diversity assessment in cassava (*Manihot esculenta* Crantz). Mol. Breeding 23:669-684.

Keim P., Kalif A., Schupp J., Hill K., Travis S., Richmond K., Adair D., Hugh-Jones M., Kuske C., and Jackson P. 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. J. Bacteriol. 179:818-824.

Kelley, M. 2006. Infectious Disease Research and Dual-Use. Ethics Journal of the American Medical Association, 8:230-234.

Kim N.-S., Park N., II, Kim S.-H., Kim S.-T., Han S.-S., and Kang K.-Y. 2000. Isolation of TC/AG repeat microsatellite sequences for fingerprinting rice blast fungus and their possible horizontal transfer to plant species. Mol. Cells 10:127-134.

Kohnemann S., Sibbing U., Pfeiffer H., and Hohoff C. 2008. A rapid mtDNA assay of 22 SNPs in one multiplex reaction increases the power of forensic testing in European Caucasians. Int .J. Legal Med. 122:517-523.

LaFramboise T. 2009. Single nucleotide polymorphism arrays: a decade of biological, computational and technological advances. Nucleic Acids Res. 1-13.

Levings III C.S. 1990. The Texas cytoplasm of maize: cytoplasmic male sterility and disease susceptibility. Science 250:942-947.

Li J., Chu X., Liu Y., Jiang J.-H., He Z., Zhang Z., Shen G., and Yu R.-Q. 2005. A colorimetric method for point mutation detection using high-fidelity DNA ligase. Nucleic Acids Res. 33:e168.

Link V.H., Powelson M.L., and Johnson K.B. 2002. Oomycetes. The Plant Health Instructor.

Lucas D.M. 1989. The ethical responsibilities of the forensic scientist: exploring the limits. J. Forensic Sci. 34:719-729.

Madden, L. V., and M. Wheelis. 2003. The threat of plant pathogen as weapons against U.S. crops. Annu. Rev. Phytopathol. 41: 155-76.

Marshall J.W. 1998. Potato cyst nematodes (*Globodera* species) in New Zealand and Australia, in: R. J. Marks and B. B. Brodie (Eds.), Potato cyst nematodes, biology, distribution and control, CAB International, Wallingfor, UK. pp. 353-394.

McKinney, H. H. 1949. Tests of varieties of wheat, barley, oats, and corn for reaction to what streak mosaic viruses. Plant Dis. Rep. 33:359-369.

Miller, J. M. 2006. The select agent rule and its impact on clinical laboratories. Clinical Microbiology Newsletter, 28:57-63.

Miller, M. T. 2005. Chapter 10: Crime Scene Investigation. p. 167-188. *In* S. H. James and J. J. Nordby (ed.), Forensic Science: An introduction to scientific investigative techniques. 2nd edition. CRC Press, Boca Raton, Florida.

Mitchelson K.R., and Moricca S. 2008. DNA fingerprinting methods for microbial pathogens: application to diagnostics, taxonomy and plant disease management, in: A. Ciancio and K. G. Mukerji (Eds.), Integrated management of diseases caused by fungi, phytoplasma and bacteria, Springer Netherlands. pp. 333-364.

Monke J. 2006. Agroterrorism: Threats and Preparedness. CSR Report for Congress Order Code RL32521:66.

Murch, R. S. 2003. Microbial forensics: building a national capacity to investigate bioterrorism. Biosecur. Bioterror. 1:117-122.

Murphy S.J., Hughes A.E., Patterson C.C., Anderson L.A., Watson R.G.P., Johnston B.T., Comber H., McGuigan J., Reynolds J.V., and Murray L.J. 2007. A population-based

assocation study of SNPs of *GSTP1, MnSOD, GPX2* and Barrett's esophagus and esophageal adenocarcinoma. Carcinogenesis 28:1323-1328.

Murray, G. M., D. Knihinicki, K. Wratten, and J. Edwards. 2005. Wheat streak mosaic and the wheat curl mite. NSW Department of Primary Industries, Primefact 99.

Netzel, L. R. 2005. Chapter 12: The forensic laboratory. p. 219-236. *In* S. H. James and J. J. Nordby (ed.), Forensic Science: An introduction to scientific investigative techniques. 2nd edition. CRC Press, Boca Raton, Florida.

Neufeld P. 2000. Legal and ethical implications of post-conviction DNA exonerations. New Eng. L. Rev. 35:639.

Niblett, C. L., K. R. Zagula, L. A. Calvert, T. L. Kendall, D. M. Stark, C. E. Smith, R. N. Beachy, and S. A. Lommel. 1991. cDNA cloning and nucleotide sequence of the *Wheat streak mosaic virus* capsid protein gene. J. Gen. Virol. 72: 499-504

Nicholson P., Simpson D.R., Weston G., Rezanoor H.N., Lees A.K., Parry D.W., and Joyce D. 1998. Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. Physiol. Mol. Plant P. 53:17-37.

Nordby, J. J. 2005. Chapter 1: Here we stand: what a forensic scientist does. p. 1-14. *In* S. H. James and J. J. Nordby (ed.), Forensic Science: An introduction to scientific investigative techniques. 2nd edition. CRC Press, Boca Raton, Florida.

Norton N., Williams N.M., Williams H.J., Spurlock G., Kirov G., Morris D.W., Hoogendoorn B., Owen M.J., and O'Donovan M.C. 2002. Universal, robust, highly quantitative SNP allele frequency measurement in DNA pools. Hum. Genet. 110:471-478.

O Grada, C. 1995. The Great Irish Famine. Cambridge University Press, New York, NY.

Osborne, D. J. 2005. A short history of warfare emphasizing the biological aspects. p. 1-8. *In* J. I. Cooper, T. Kuehne, V. P. Polischuk(ed.), Virus Diseases and Crop Biosecurity. Springer, Dordrecht, The Netherlands.

Ovesna J., Polakova K., Kucera L., and Rulcova J. 2003. SNP typing in cereals: comparison of SSCP and SNaPshot markers using Barley *Mlo* locus as a model. Czech J. Genet. Plant Breed. 39:109-112.

Oxford Dictionary of English (revised edition). (2005) bioterrorism. C. Soanes, A. Stevenson (ed.), Oxford University Press. http://www.oxfordreference.com/views/ENTRY.html?subview=Main&entry=t140.e7436 . Accessed 27 November 2007.

Pollack S. 1973. Observations on the adversary system and the role of the forensic scientist - 'scientific truth' v. 'legal truth'. J. Forensic Sci. 18:173-177.

Quintans B., Alvarez-Iglesias V., Salas A., Phillips C., Lareu M., and Carracedo A. 2004. Typing of mitochondrial DNA coding region SNPs of forensic and anthropological interest using SNaPshot minisequencing. Forensic Sci. Int. 140:251-257.

Rabenstein, F., D. L. Seifers, J. Schubert, R. French, and D. C. Stenger. 2002. Phylogenetic relationships, strain diversity and biogeography of tritimoviruses. J. Gen. Virol. 83: 895-906.

Ruffell A., and McKinley J. 2005. Forensic geoscience: applications of geology, geomorphology and geophysics to criminal investigations. Earth-Science Reviews 69:235-247.

Sanchez-Sanchez, H., Henry, M., Cardenas-Soriano, E., and Alvizo-villasana, H. F. 2001. Identification of *Wheat streak mosaic virus* and its vector *Aceria tosichella* in Mexico. Plant Dis.85:13-17.

Schaad N.W., Opgenorth D., and Gaush P. 2002. Real-Time Polymerase Chain Reaction for One-Hour On-Site Diagnosis of Pierce's Disease of Grape in Early Season Asymptomatic Vines. Phytopathology 92:721-728.

Schaad N.W., Frederick R.D., Shaw J., Schneider W.L., Hickson R., Petrillo M.D., and Luster D.G. 2003. Advances in molecular-based diagnostics in meeting crop biosecurity and pytosanitary issues. Annu. Rev. Phytopathol. 41:305-324.

Scientific Working Group for the Analysis of Seized Drugs. 2003. Forensic Science Communications. 7.

Scott A.M. 2009a. Crime Scene Investigation. John Wiley & Sons, Ltd.

Scott A.M. 2009b. Crime Scene Documentation. John Wiley & Sons, Ltd.

Shukla, D., Frenkel, M., and Ward, C. 1991. Structure and function of the potyvirus genome with special reference to the coat protein coding region. Can. J. Plant Path. 13:178-191.

Signoret, P. 1974. Virus diseases of Gramineae in Southern France. Microbiologija, 11:115-120.

Sill, W. H. J., and Connin, R. V. 1953. Summary of the known host range of the *Wheat streak mosaic virus*. Transactions of the Kansas Academy of Science 56:411-417.

Smith G.B., and Gordon J.A. 1996. The admission of DNA evidence in state and federal courts. Fordham L. Rev. 65:2465.

Sorg, M. H. 2005. Chapter 7: Forensic anthropology. p. 99-118. *In* S. H. James and J. J. Nordby (ed.), Forensic Science: An introduction to scientific investigative techniques. 2nd edition. CRC Press, Boca Raton, Florida.

Speijer H., Savelkoul P.H.M., Bonten M.J., Stobberingh E.E., and Tjhie J.H.T. 1999. Application of different genotyping methods for *Pseudomonas aeruginosa* in a setting of endemicity in an intensive care unit. J. Clin. Microbiol. 37:3654-3661.

Stack J., Cardwell K., Hammerschmidt R., Byrne J., Loria R., Snover-Clift K., Baldwin W., Wisler G., Beck H., Bostock R.M., Thomas C.S., and Luke E. 2006. The National Plant Diagnostic Network. Plant Dis. 90:128-136.

Stack J., Hammerschmidt R., Hudler G., Luke E., and Bostock R. 2007. National Plant Diagnostic Network: a record of accomplishment, CSREES five-year review.

Starrs J.E. 1991. The forensic scientist and the open mind. Journal of the Forensic Science Society 31:111-149.

Stenger, D. C., D. L. Seifers, and R. French. 2002. Patterns of polymorphism in *Wheat streak mosaic virus*: Sequence space explored by a clade of closely related viral genotypes rivals that between the most divergent strains. Virology. 302: 58-70.

Stenger, D. C., Hein, G. L., Gildow, F. E., Horken, K. M., and French, R. 2005a. Plant virus HC-Pro is a determinant of eriophyid mite transmission. J. Virol. 79:9054-9061.

Stenger, D. C., French, R., and Gildow, F. E. 2005b. Complete deletion of *Wheat streak mosaic virus* HC-Pro: a null mutant is viable for systemic infection. J. Virol. 79:12077-12080.

Stenger, D. C., Young, B. A., and French, R. 2006a. Random mutagenesis of *Wheat streak mosaic virus* HC-Pro: non-infectious interfering mutations in a gene dispensable for systemic infection of plants. J. Gen. Virol . 87: 2741-2747.

Stenger, D. C., Hein, G. L., and French, R. 2006b. Nested deletion analysis of *Wheat streak mosaic virus* HC-Pro: Mapping of domains affecting polyprotein processing and eriophid mite transmission. Virology *350:*465-474.

Stenger D.C., Young B.A., Qu F., Morris T.J., and French R. 2007 *Wheat streak mosaic virus* lacking helper component-proteinase is competent to produce disease synergism in double infections with *Maize chlorotic mottle virus*. Phytopathology 97:1213-1221.

Strange, R. N., and P. R. Scott. 2005. Plant disease: A threat to global food security. Annu. Rev. Phytopathol. 43: 83-116.

Stromberg, E. L. 2003. Wheat streak mosaic. Virginia Tech. http://www.ppws.vt.edu/stromberg/smallgrain/biology/wsmv.html

Tatum L.A. 1971. The southern corn leaf blight epidemic. Science 171:1113-1116.

Taylor M.E. 1973. Scanning electron microscopy in forensic science. Journal of the Forensic Science Society 13:269-280.

Thomas, J. A., and Hein, G. L. 2003. Influence of volunteer wheat plant condition on movement of the wheat curl mite, *Aceria tosichella*, in winter wheat. Exp. Appl. Acarol. 31:253-268.

Townsend, L., D. Johnson, and D. Hershman. 1996. *Wheat streak mosaic virus* and the wheat curl mite. University of Kentucky, ENTFACT-117. http://www.ca.uky.edu/entomology/entfacts/ef117.asp

Trestrail J.H. 2007. Crime Scene Investigation, Criminal Poisoning, Humana Press. pp. 69-81.

Tucker, J. B., and Koblentz, G. D. 2009. The four faces of microbial forensics. Biosecur. Bioterror. 7:389-397.

Ullstrup A.J. 1972. The impacts of the southern corn leaf blight epidemics of 1970-1971. Annu. Rev. Phytopathol. 10:37-50.

Van K., Hwang E.-Y., Kim M.Y., Park H.J., Lee S.-H., and Cregan P.B. 2005. Discovery of SNPs in Soybean Genotypes Frequently Used as the Parents of Mapping Populations in the United States and Korea. J. Hered. 96:529-535..

Wang D.G., Fan J.-B., Siao C.-J., Berno A., Young P., Sapolsky R., Ghandour G., Perkins N., Winchester E., Spencer J., Kruglyak L., Stein L., Hsie L., Topaloglou T., Hubbell E., Robinson E., Mittmann M., Morris M.S., Shen N., Kilburn D., Rioux J., Nusbaum C., Rozen S., Hudson T.J., Lipshutz R., Chee M., and Lander E.S. 1998. Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. Science 280:1077-1082.

Watling R.J., Lynch B.F., and Herring D. 1997. Use of laser ablation inductively coupled plasma mass spectrometry for fingerprinting scene of crime evidence. J. Anal. Atom. Spectrom. 12:195-203.

Weston, N. 1998. Chapter 2: The crime scene. p. 15-46. *In* P. White (ed.), Crime scene to court: the essentials of forensic science. The Royal Society of Chemistry, Cambridge, UK.

Wheelis, M., R. Casagrande, and L. V. Madden. 2002. Biological attack on agriculture: low-tech, high-impact bioterrorism. BioScience. 52: 569-576.

Wilson M.R., Stoneking M., Holland M.M., Dizinno J.A., and Budowle B. 1993. Guidelines for the use of mitochondrial DNA sequencing in forensic science. Crime Lab. Digest 20:68-77.

Wright, R. K. 2005. Chapter 4: Investigation of Traumatic Deaths. p. 43-60. *In* S. H. James and J. J. Nordby (ed.), Forensic Science: An introduction to scientific investigative techniques. 2nd edition. CRC Press, Boca Raton, Florida.

Young, B. A., Hein, G. L., French, R., and Stenger, D. C. 2007. Substitution of conserved cysteine residues in *Wheat streak mosaic virus* HC-Pro abolished virus transmission by the wheat curl mite. Arch. Virol. 152:2107-2111.

CHAPTER III

CROP BIOAGENT INTRODUCTION INTENT ASSESSMENT TOOL: ASSESSING THE LIKELIHOOD THAT A PLANT PATHOGEN INTRODUCTION WAS INTENTIONAL USING *WHEAT STREAK MOSAIC VIRUS* AS A MODEL PATHOSYSTEM

Abstract

The agricultural industry is vulnerable to plant diseases caused by a variety of abundant pathogens and, hence, can be targeted by bioterrorists or criminals seeking to disrupt agricultural systems or receive personal gain. Pathogens may go undetected for days, weeks, months, or even years due to the latent period between infection/introduction and symptom development, making detection of an intentional inoculation difficult. The Crop Bioagent Introduction Intent Assessment Tool (CBIIAT), a decision tool that uses *Wheat streak mosaic virus* (WSMV) as a model pathosystem, was developed to assist investigators in determining if a plant disease outbreak was caused by an intentional pathogen introduction. The CBIIAT was validated by seven testers of different professional and educational backgrounds, representing potential tool end-users, who applied the tool to assess the likelihood of intentional pathogen introduction in two WSMV-infected wheat fields, only one of which was the result of human introduction. By comparing the assessment values, number of criteria misinterpreted, level of preparation and participation, and educational background of the testers, I demonstrated the successful classification of each test case into the appropriate intent category regardless of the scientific

experience or previous WSMV knowledge of the tester. Neither the level of familiarity with the tool and the assessment procedure nor consultation with the Cooperative Extension Agent or producer during the assessment affected the outcome of the assessment, although these behaviors did result in a better understanding of the tool criteria. The results demonstrate that a plant disease outbreak can be assessed for likelihood of intent using the CBIIAT, which can serve as a model for the development of similar tools for other plant pathogens.

Introduction

The first critical question of any forensic investigation is, "Has a crime been committed?" This question may be easily answered in some instances, such as when a shooting results in a homicide or a store robbery is caught on tape. However, determining if a plant disease was incited intentionally, rather than occurring via natural means, may not be easy. Plant pathologists in the United States rarely consider the possibility that pathogens might be disseminated by persons having harmful intent. Recent national emphasis on homeland security, however, has highlighted the vulnerability of our crops and plant systems to such actions, leading to an awareness of the need for new strategies for agricultural biosecurity (Cameron and Pate, 2001; Casagrande, 2000; Cupp, Walker II, and Hillison, 2004; Madden and Wheelis, 2003; National Institute of Justice, 2006; Wheelis, Casagrande, and Madden, 2002).

Criminal acts targeting our crops, forest, rangelands, and other plant resources can be divided into biocrime and bioterrorism. A biocrime involves the use of a biological agent to harm an individual or a targeted group. Motives might include spite, revenge, competition, jealousy, economic gain, or the opportunity to make a statement. Bioterrorism refers to an act in which a biological agent is used to target a nation, state, or a specific area, and can be mounted by a terrorist group or a government.

Symptoms of plant diseases typically do not appear immediately after infection, but rather after a few days, weeks, or even months. This delay is one impediment to distinguishing between a natural and intentional outbreak (Fletcher et al, 2006). Others include the abundance of plant diseases that occur naturally, the genetic variability among plant pathogens of the same species, and the limited information available on the thousands of plant pathogens found in nature.

We sought to assist first responders, law enforcement personnel and forensic investigators in the determination of potential intent in a plant pathogen introduction. A decision tool, designated the Crop Bioagent Introduction Intent Assessment Tool (CBIIAT), was designed to assist responders to evaluate various features of a disease outbreak, and then weigh and quantitated these elements to yield a useful measure of intent probability. Some of the disease information required can be collected on-site, while other input can be obtained by interviewing the producer(s), Area and/or County Cooperative Extension personnel, and/or the regional plant disease diagnostician (often a person associated with the state's branch of the USDA's National Plant Diagnostic Network**)** (Stack et al, 2006). The tool consists of a series of statements, each weighted based on relevance to the determination of human intent. The evaluator assesses the applicability of each statement, assigning a numerical rating. A formula is then applied to yield an estimate of the likelihood that the disease event was human-incited. The CBIIAT was modeled after a similar tool developed for application to an outbreak of the human disease, tularemia, in Kosovo in 1999 (Grunow and Finke, 2002).

Plant pathogens are diverse, and include bacteria, fungi, viruses, viroids, nematodes, protozoans and parasitic plants. An ideal decision tool should be applicable to disease caused by any pathogen, but this CBIIAT was designed around a model pathosystem involving *Wheat streak mosaic virus* (WSMV) and its primary host, wheat. Wheat is the principal grain grown in the Unites States, with approximately 63 million acres planted each year (National Agricultural

Statistics Service, 2010). WSMV occurs in most wheat producing areas of the U.S. on an annual basis and can lead to yield losses as high as 100% in infected fields (Christian and Willis, 1993).

WSMV serves as a good model because its prevalence in Oklahoma made it convenient to access naturally affected wheat fields, and possible to purposefully inoculate a field plot to simulate a criminal event. Furthermore, the epidemiology and genetics of the virus have been studied extensively. WSMV is a positive sense, single-stranded RNA virus belonging to the genus *Tritimovirus* within the family *Potyviridae*, one of the largest families of plant viruses (Stenger, Seifers, and French, 2002). A distinctive disease symptom is the mosaic pattern on the leaves; light green streaks that appear parallel to the leaf veins, later turning yellow, creating green and yellow blotches (Murray et al, 2005). Stunting, wheat head sterility, and premature plant death may also occur during severe outbreaks (Stromberg, 2003).

WSMV can be spread from plant to plant by its only known vector, *Aeceria tosichella* Keifer, the wheat curl mite (WCM). This arthropod feeds not only on wheat, the primary host of WSMV, but also on many other cereals and grasses, most of which are also hosts of WSMV (Murray et al, 2005). The WCM is microscopic (0.2 mm long) and relies on the wind or other insects to move it from plant to plant, resulting in a pattern of virus spread that correlates with wind direction (Slykhuis, 1955; Somsen and Sill, 1970).

Both WSMV and the WCM require a living host for their survival. Before wheat is harvested, the mites move onto new host plants, often volunteer wheat or a different crop, such as corn. This "green bridge" serves as a source of inoculum for the next wheat crop (Somsen and Sill, 1970; Thomas and Hein, 2003; Thomas, Hein, and Lyon, 2004).

This report describes the development, evaluation and application of a decision tool designed to assist investigators in determining if a WSMV outbreak was caused naturally or by human activity. The CBIIAT's utility and accuracy was evaluated by multiple testers of diverse

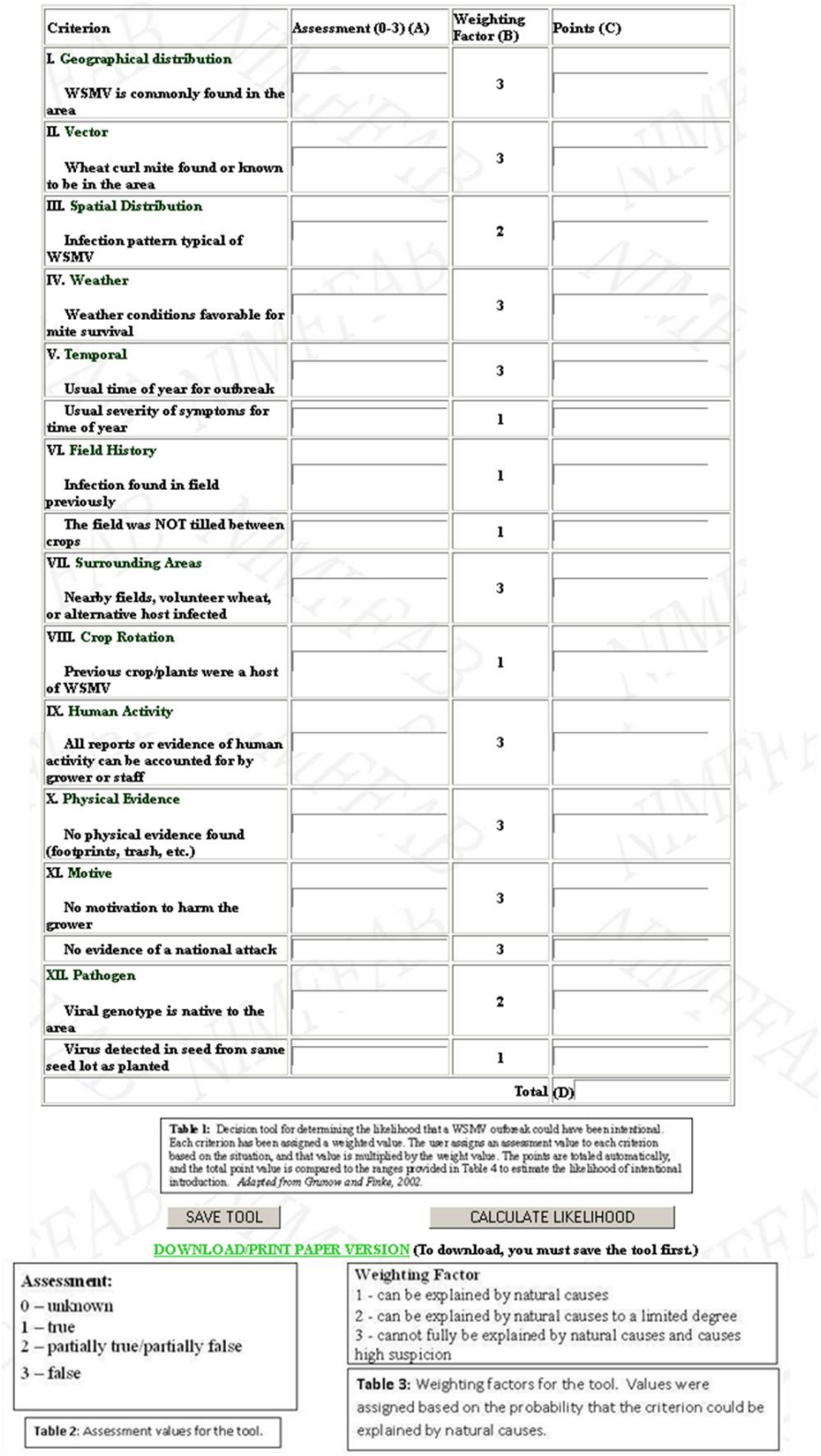
professional backgrounds and training levels. Tester input was incorporated into the final version of the tool, which is posted on the World Wide Web (http://bioinfosu.okstate.edu/NIMFFAB). In its current form, the CBIIAT may be valuable in forensic investigations of plant disease outbreaks, but its primary utility is to serve as a model for the development of new decision tools for plant disease outbreaks caused by any plant pathogen on any host.

Materials and Methods

I – Decision tool development

I - a - Criterion Selection

Criteria to be included in the tool (Figure 1), which were selected by reviewing WSMV literature and by consulting experts on WSMV epidemiology and forensic science, include various factors related to disease development, the pathogen, social and political factors, and traditional forensic issues. The utility of each criterion in the CBIIAT, and the overall effectiveness of the tool, were evaluated over two years (2008, 2009) in Oklahoma wheat fields.



Criterion	Assessment (0-3) (A)	Weighting Factor (B)	Points (C)
I. Geographical distribution WSMV is commonly found in the area		3	
II. Vector Wheat curl mite found or known to be in the area		3	
III. Spatial Distribution Infection pattern typical of WSMV		2	
IV. Weather Weather conditions favorable for mite survival		3	
V. Temporal Usual time of year for outbreak		3	
Usual severity of symptoms for time of year		1	
VI. Field History Infection found in field previously		1	
The field was NOT tilled between crops		1	
VII. Surrounding Areas Nearby fields, volunteer wheat, or alternative host infected		3	
VIII. Crop Rotation Previous crop/plants were a host of WSMV		1	
IX. Human Activity All reports or evidence of human activity can be accounted for by grower or staff		3	
X. Physical Evidence No physical evidence found (footprints, trash, etc.)		3	
XI. Motive No motivation to harm the grower		3	
No evidence of a national attack		3	
XII. Pathogen Viral genotype is native to the area		2	
Virus detected in seed from same seed lot as planted		1	
		Total (D)	

Table 1: Decision tool for determining the likelihood that a WSMV outbreak could have been intentional. Each criterion has been assigned a weighted value. The user assigns an assessment value to each criterion based on the situation, and that value is multiplied by the weight value. The points are totaled automatically, and the total point value is compared to the ranges provided in Table 4 to estimate the likelihood of intentional introduction. *Adapted from Grunow and Finke, 2002.*

SAVE TOOL

CALCULATE LIKELIHOOD

DOWNLOAD/PRINT PAPER VERSION **(To download, you must save the tool first.)**

Assessment:
0 – unknown
1 – true
2 – partially true/partially false
3 – false

Table 2: Assessment values for the tool.

Weighting Factor
1 - can be explained by natural causes
2 - can be explained by natural causes to a limited degree
3 - cannot fully be explained by natural causes and causes high suspicion

Table 3: Weighting factors for the tool. Values were assigned based on the probability that the criterion could be explained by natural causes.

Figure 1: Screenshot of the CBIIAT assessment table, including the assessment values and weighting factor descriptions. The criteria are the revised statements after completion of the evaluations. Adapted from Grunow and Finke, 2002.

I - b - Weighting Factors

Each criterion was assigned a weighting value from '1' (low) to '3' (high) based on information gathered from scientific literature and WSMV epidemiologists, reflecting its perceived level of influence on the question of whether the outbreak was due to intentional human activity. For example, a weighting factor of '3' was assigned to the vector criterion (Figure 1). WSMV is disseminated in nature only by the arthropod vector, the wheat curl mite. If the mite is not found and has not been reported to occur in the area of a WSMV infection, the presence of WSMV cannot be fully explained by natural causes. In contrast, a weighting factor of '1' was assigned to the field history criterion; even if WSMV had not been found in the same field in previous years, its presence can still be explained by natural causes, such as a possible introduction from nearby fields or wild grasses.

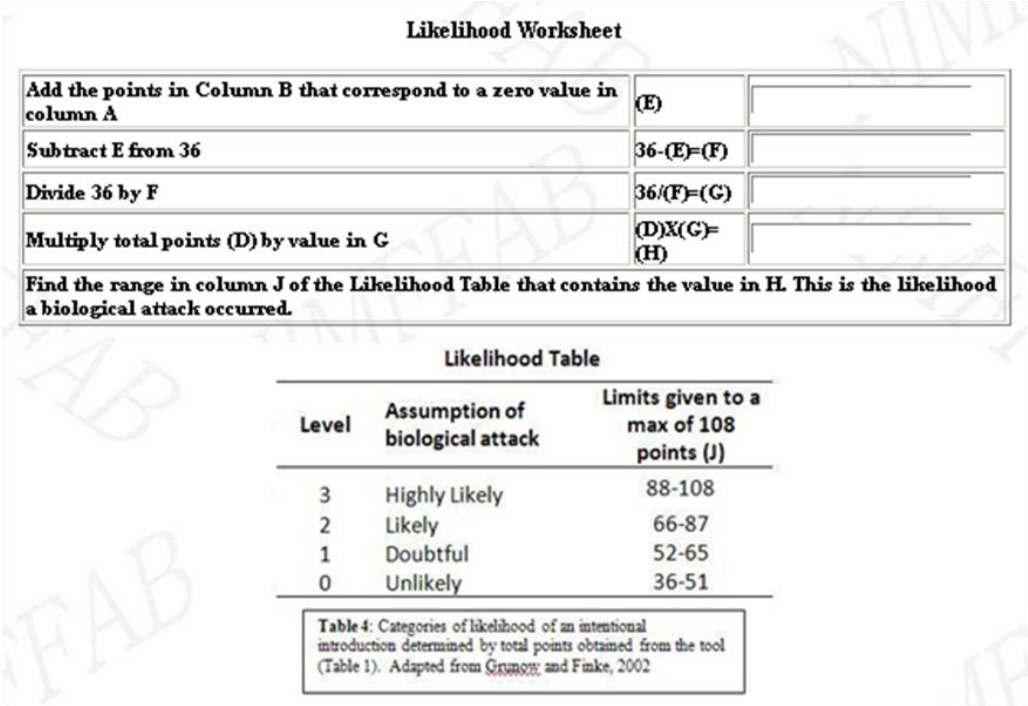
I - c - Assessment Values

To estimate the likelihood that a WSM outbreak was intentional, the user is directed to assign to each tool criterion an assessment value based on applicability of the statement with respect to the incident (Figure 1). If a statement is completely applicable, it should receive an assessment value of '1'. If only partially applicable, the user would designate an assessment value of '2'; if the statement is false, it would receive a value of '3'. If no data are available on a particular criterion, or if it is not relevant to the situation, an assessment value of '0' may be given.

I - d - Likelihood Calculation

After the user assigns an assessment value to each criterion in Figure 1, the likelihood that a disease event was human-incited can be calculated. The rating for each criterion is determined by multiplying the user's assessment value by the fixed weighting factor. The total number of points for all criteria are summed and compared to the point value ranges in the

Likelihood Table in Figure 2, which indicate the likelihood that an introduction was intentional. The values in the Likelihood Table are based on the total possible points for the tool. However, if a '0' is assigned as an assessment value, the ranges in the Likelihood Table must be adjusted to compensate for the omission of a criterion, by inputting the summed value of points from the assessment table in Figure 1 into the Likelihood Worksheet (Figure 2). The final point value calculated from the Likelihood Worksheet is then compared to the value ranges in the Likelihood Table to determine the likelihood that a pathogen introduction was intentional.



Likelihood Worksheet

Add the points in Column B that correspond to a zero value in column A	**(E)**	
Subtract E from 36	**36-(E)=(F)**	
Divide 36 by F	**36/(F)=(G)**	
Multiply total points (D) by value in G	**(D)X(G)=(H)**	
Find the range in column J of the Likelihood Table that contains the value in H. This is the likelihood a biological attack occurred.		

Likelihood Table

Level	Assumption of biological attack	Limits given to a max of 108 points (J)
3	Highly Likely	88-108
2	Likely	66-87
1	Doubtful	52-65
0	Unlikely	36-51

Table 4: Categories of likelihood of an intentional introduction determined by total points obtained from the tool (Table 1). Adapted from Grunow and Finke, 2002

Figure 2: Screenshot of the online tool showing the worksheet and table used to calculate the likelihood of an intentionally introduced outbreak.

I - e - Likelihood Table

The Likelihood Table in Figure 2 provides point value ranges corresponding to four levels of probable intent: highly likely, likely, doubtful, and unlikely. Each level corresponds to a percentage of the total possible points (100-70%, 69-40%, 39-20%, 19-0%, respectively). These

ranges were determined by identifying the final point values obtained through CBIIAT assessments of both naturally and intentionally introduced WSM outbreaks.

II – Development of supporting documents

Many of the targeted end-users of this tool, such as law enforcement officers, producers, and Cooperative Extension personnel, may be unfamiliar with WSM and its causal virus. To assist users in understanding and assigning assessment values to each criterion, a WSMV fact sheet, generated by consulting relevant scientific literature related to each criterion, was included in the CBIIAT (Appendix A). Further, examples of assessments are provided in each section of the fact sheet to illustrate how such factors may impact the assessment.

Because the CBIIAT was designed to be used in an investigative setting in which extensive incident documentation would be necessary, a report worksheet was also developed. This worksheet, which was adapted from the Oklahoma State University Plant Disease and Insect Diagnostic Laboratory (OSU PDIDL) sample submission report, provides space to record the reporting agency, the field history and conditions, weather, pathogen and disease components, laboratory test results and forensically relevant physical evidence. Completion of the worksheet is not required for CBIIAT use, but can assist in assessing and documenting the incident.

III –Field selection

Two natural WSM outbreaks, one in central and the other in western Oklahoma, were identified via surveillance reports to the OSU PDIDL. WSMV positive fields, determined through ELISA and PCR (performed by the OSU PDIDL), were located in Kingfisher and Canadian Counties in 2008, in Canadian County in 2009, and in Texas County in 2010. Assessments were conducted by visiting the fields and by consulting with the area extension agent and producer when they were available.

To create an intentionally caused outbreak, wheat was planted at the OSU Plant Pathology Research Farm in Stillwater, OK in the fall of 2008 and 2009 and was intentionally inoculated with WSMV. A laboratory-maintained isolate of WSMV (originally collected from infected wheat in Oklahoma; specific origin unknown) was spray inoculated onto month old winter wheat, *Triticum aestivum* 'Vona', as described by Hunger et al (1992). Forty plots (four m^2) scattered throughout a 361 m^2 field were inoculated to create a random distribution of the disease.

IV – Online tool development

To enhance the accessibility of the tool to law enforcement and other end-users the CBIIAT was adapted to an online format that provides a fully interactive interface (http://bioinfosu.okstate.edu/NIMFFAB). After registering to gain access, the user enters their assessment values into the appropriate spaces and the likelihood calculation is performed automatically. Specific information needed for each criterion, provided in the factsheet, is available by clicking a link from the assessment table to disease specific information. Other resources, including the plant disease worksheet, a glossary of terms, example assessments, and links to other informative websites also are available. A report of the completed assessment, including the calculated likelihood, can be printed and saved.

V – Decision tool evaluation

To evaluate the effectiveness of the CBIIAT in determining the likelihood that a disease event was either naturally or intentionally/human-incited, assessments were completed by testers representing various groups of potential end-users, on both a naturally occurring and an intentionally inoculated WSMV outbreak in Oklahoma in 2010. At each field site, seven individuals of different backgrounds, who might be involved in an authentic case investigation,

were recruited as testers. Due to limitations of travel, only three of the seven were able to evaluate both fields. The testers included research plant pathologists, Cooperative Extension agents, wheat producers and law enforcement officers. Each tester had access to the CBIIAT several days in advance and was encouraged to familiarize himself with the tool and the process. While the testers were in the field, the producer and/or County Cooperative Extension agent responsible for the area were available to answer questions about the incident or field. The number of clarifying or informational questions asked by each tester was recorded. Testers were asked also if they had familiarized themselves with the tool before arriving at the site and whether they had consulted the factsheet during their assessment. The final point values reached by each tester and the data collected about their assessments were used to assess the effectiveness of the tool in assisting a disease incident investigator to judge the likelihood that the event could have been caused intentionally.

Results

I – Creation of tool

The CBIIAT was created with inclusion of criteria that are relevant to the determination of intent of a WSM disease outbreak and cover multiple elements of an outbreak. The content and composition appear to be appropriate for assessing introduction intent after preliminary evaluations at various Oklahoma WSM outbreaks. The online CBIIAT was created with user-friendly options and examples. The online version will be used to disseminate the tool to law enforcement personnel and first responders.

II– Determination of intent likelihood

The range of total point values reached by the testers ranged from 36 to 87. Despite the variation, all totals fell within the point ranges that correctly assessed the intent of the two outbreaks; ‘doubtful’ or ‘unlikely’ for the naturally infected field and ‘likely’ or ‘highly likely’

for the intentional field (Figure 3). Therefore, all the testers arrived at appropriate conclusions in each test case about the likelihood that the disease was either naturally or intentionally incited.

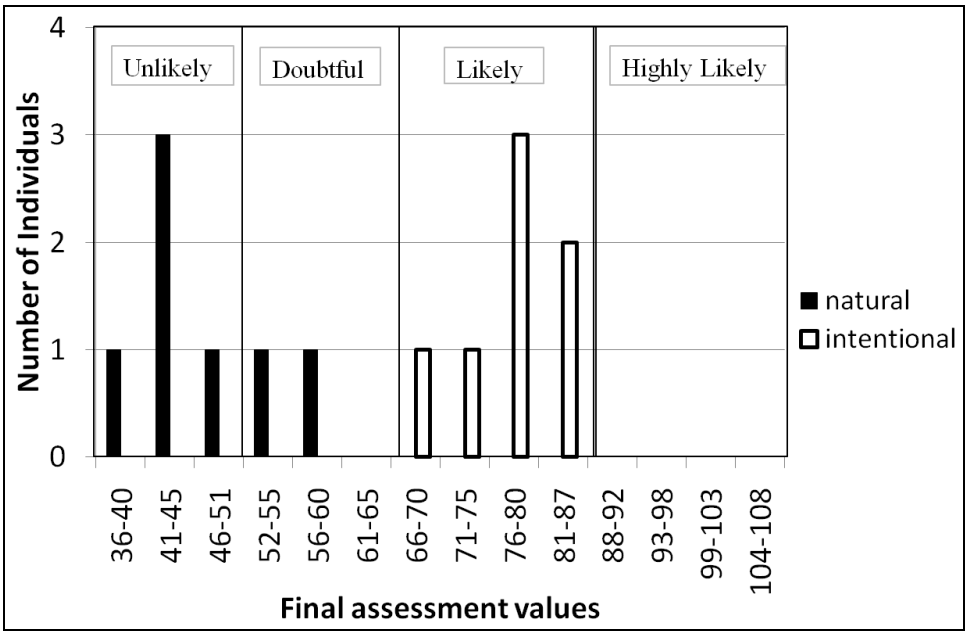


Figure 3: Distribution of tester assessment values for both the natural and intentional wheat streak mosaic outbreaks. The categories across the top indicate the likelihood category that corresponds to the assessment values (shown in Figure 2).

III – Familiarity with the tool may contribute to a realistic assessment

Due to the complexity of the tool format and the fact that investigators from law enforcement agencies would likely be unfamiliar with WSMV, I hypothesized that becoming familiar with the tool, understanding how the assessment works, and taking advantage of the resources that were available during the assessment would increase a users' ability to interpret the scenario and lead to a more accurate assessment. To test this hypothesis, the number of criteria misinterpreted by each tester was determined by reviewing their assessment choices and identifying those criteria for which they assigned inappropriate assessment values (Table 1). Furthermore, as each tester performed their assessment, notes were recorded on their use of resources. The number of criteria misinterpreted by each tester during the assessment was compared to the final point value they obtained. The testers' familiarity with the tool prior to the

assessment was also considered. Most criteria were misinterpreted by at least one tester, but three criteria were misinterpreted by five or more testers. The misinterpretation was most likely due to compounded negatives concerning whether a 'false' assessment should be assigned or specialized scientific language in the criterion statement. The wording of these criteria was revised to facilitate tester interpretation (compare assessment table in Figure 1 to Table 1).

Table 1:Number of times each criterion was misinterpreted by the testers

CRITERION	# OF MISINTERPRETATIONS*
I. Geographical distribution	
WSMV is commonly found in the area	3
II. Vector	
Wheat curl mite found in the area	3
III. Spatial Distribution	
Infection pattern typical of WSMV	2
IV. Weather	
Weather conditions favorable for mite survival	0
V. Temporal	
Usual time of year for outbreak	1
Usual severity of symptoms for time of year	0
VI. Field History	
Infection found in field previously	0
The field was not tilled between crops	5
VII. Surrounding Areas	
Nearby fields, volunteer wheat, or alternative host infected	0
VIII. Crop Rotation	
Wheat rotated with host of WSMV	2
IX. Human Activity	
No unusual human activity present or reported	5
X. Physical Evidence	
No physical evidence found at scene	3
XI. Motive	
No motivation to harm the grower	1
No evidence of a national attack	2
XII. Pathogen	
virus strain is native to the area	7
Virus detected in seed from same seed as planted	2

* Out of 14 assessments

Figure 4 shows the distribution of final point values as they relate to the number of criteria misinterpreted and whether or not the tester previewed the tool. The number of criteria misinterpreted appears to be related to the tester's familiarity with the tool prior to the assessment. More criteria were misinterpreted by testers who had not previewed the tool than by

those who did so. To determine if the appropriateness of a tester's assessment was affected by criteria misinterpretation, the absolute value of the deviation from the true value (final point value received when the assessment was completed as designed) was plotted against the number of misinterpreted criteria (Figure 5). While testers having the greatest number of misinterpreted criteria appear to show the greatest deviation from the true value, the correlation is weak if present at all. Therefore, although studying the tool prior to assessment appears to decrease the number of criteria misinterpreted, it may not enhance the accuracy of the assessment.

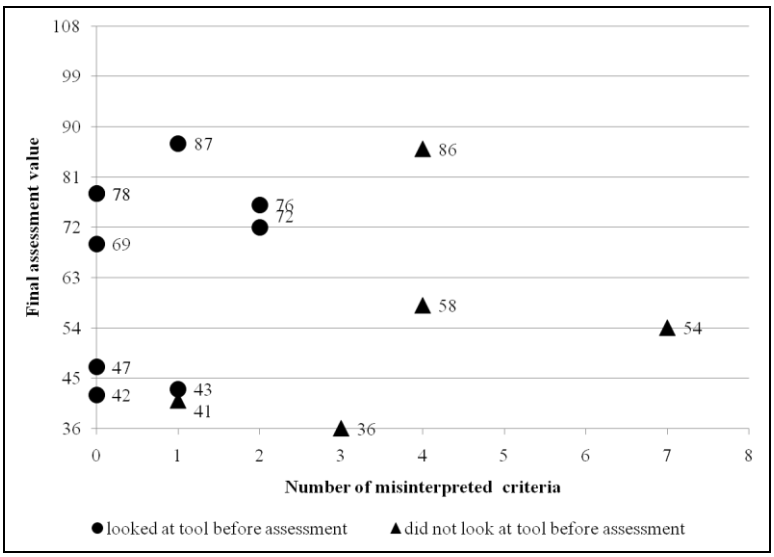


Figure 4: Distribution of the tester's final assessment values for the natural and intentional outbreak assessments in relation to the number of misinterpreted criteria. The testers with the fewest misinterpreted questions became familiar with the tool prior to assessment.

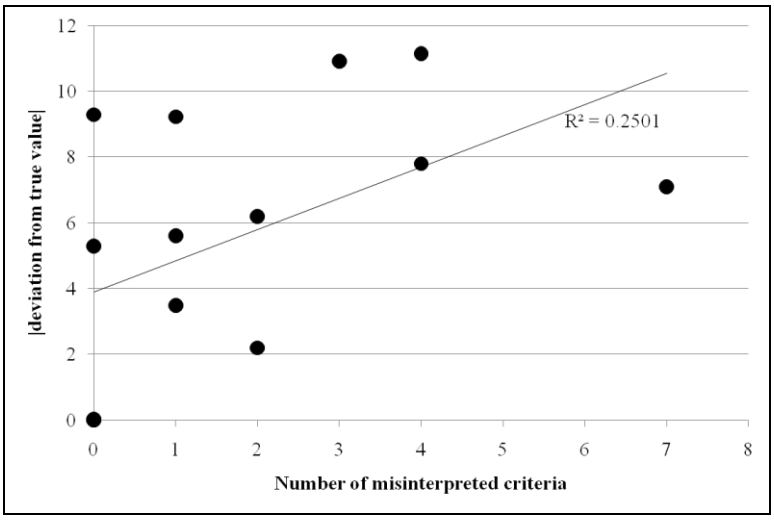


Figure 5: Correlation between the deviation from the true assessment value and the number of misinterpreted criteria for all the testers. The R^2 value indicates there is a weak correlation.

IV – Enhancing the assessment through conversations with the grower and Cooperative Extension agent.

To obtain all the information needed to complete the assessment using the CBIIAT, it was necessary for the tester to question the extension agent and/or producer of the wheat. Asking questions during the assessment may provide testers a better understanding of the situation and lead to a more insightful assessment of the outbreak. To determine whether the number of tester questions asked influenced the assessment outcome, the number of questions was plotted against the absolute value of the deviation from the true assessment value (final point value received when assessment was completed as designed) (Figure 6). There was no correlation between the number of questions asked and the accuracy of the assessment. However, with few exceptions, the number of questions asked was negatively correlated with the number of criteria misinterpreted (Figure 7).

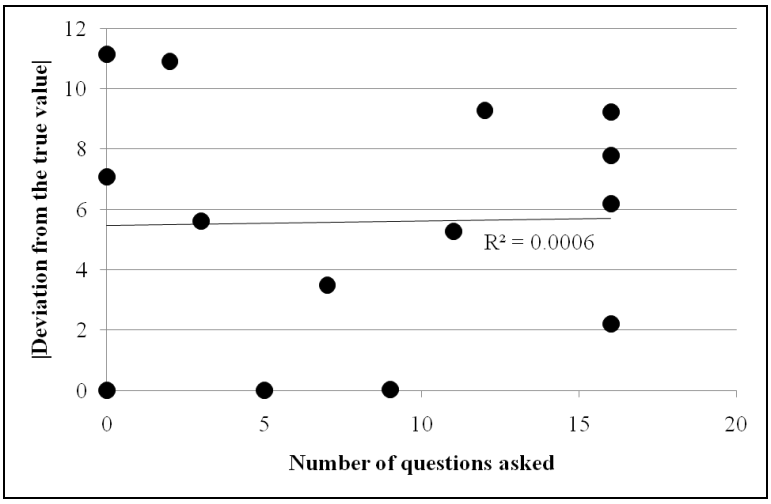


Figure 6: Correlation between the deviation from the true assessment value and the number of questions each tester asked during the outbreak assessments. The R^2 value indicates there is no correlation between the two.

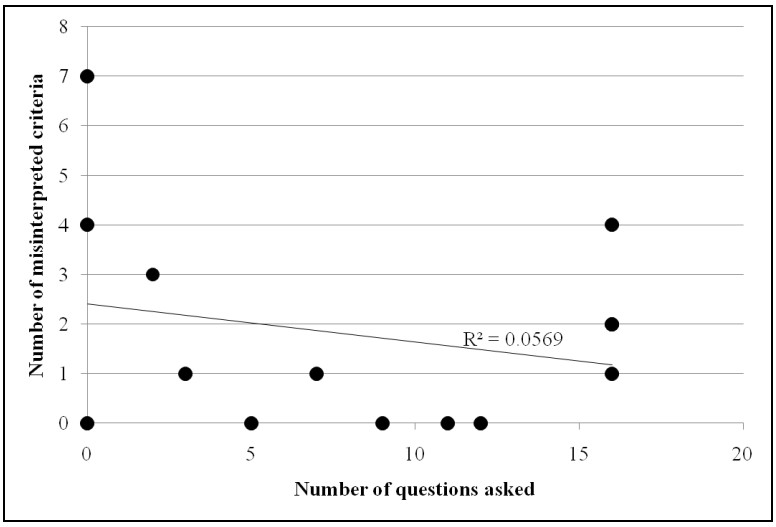


Figure7: Correlation between the number of misinterpreted criteria and the number of questions each tester asked during the outbreak assessment. The R^2 value indicates there is no correlation between the two.

V- An appropriate assessment was achieved regardless of tester educational background.

The purpose of the CBIIAT is to assist outbreak investigators who lack experience in plant pathology or science. To determine whether the tool was developed so that individuals of diverse backgrounds and education could apply the tool appropriately, the final assessment values reached by each tester (scientist vs. non-scientist) were compared to the number of criteria misinterpreted (Figure 8). Scientists and non-scientists each clustered into their respective groups, with the non-scientists misinterpreting more criteria than the scientists, but the ranges of final point values assigned by scientist and non-scientist testers for the intentional outbreak overlapped. In contrast, the non-scientists reached higher assessment values for the natural outbreak than the scientists did. Even with the slight difference in assessment values, however, all the individuals came to similar conclusions about the natural outbreak (Figure 3). Therefore, the outcome of the assessment was not affected by whether the tester performing the assessment had a scientific education.

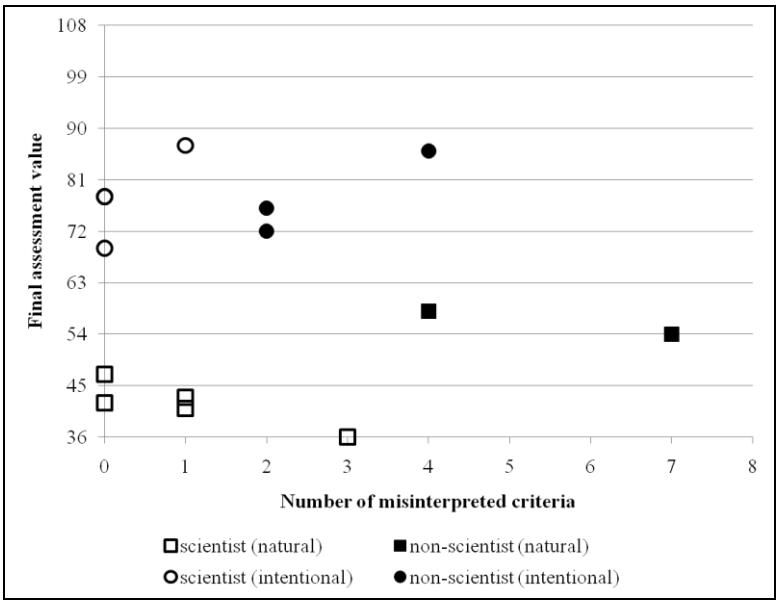


Figure 8: Distribution of the tester's final assessment values in relation to the number of misinterpreted criteria. The shapes indicate the tester's scientific experience, showing the scientist had the fewest number of misinterpreted criteria for both the natural and intentional outbreaks.

Discussion

Each occurrence of a particular plant disease in a particular crop host will have unique features, but some characteristics will be similar each time and can be utilized in decision making. For WSMV, the essential role of the wheat curl mite for natural dissemination presents a characteristic pattern of virus spread within a field; the most severely affected wheat will generally occur at the site of mite introduction, with an infection gradient extending through the field in the direction of predominant winds. Between growing seasons, the mites survive on volunteer wheat or alternative grassy hosts, i.e. the "green bridge." In a typical WSM outbreak, an alternative grassy host species or an alternative host such as corn can often be identified near the point of introduction. Introduction of the virus from a nearby field can be discerned by comparing the locations of infected plants to wind direction.

The intentionally inoculated wheat field used in this study differed significantly from the natural outbreaks. The location of the infected field was unusual for a WSMV outbreak, and there were no other WSMV infected fields nearby. The symptoms were sporadic throughout the

field and did not resemble the spread pattern of an introduction of the virus through a wind-blown arthropod vector. The affected plants were severely symptomatic with almost a 100% yield loss, a loss rate that occurs rarely in a natural outbreak. Because the intentionally inoculated field was located on the OSU Plant Pathology Research Farm, it is possible that the testers guessed the virus was introduced by humans. However, the testers were not informed of the intent prior to the assessment, nor were they informed they would be assessing an intentionally inoculated field. Therefore, the probability of the testers guessing the intent was low.

Although the CBIIAT was designed for use by members of law enforcement agencies, it could be beneficial also to plant disease diagnosticians, Cooperative Extension personnel, or producers. Each potential user group may have different perspectives and training backgrounds. Therefore, it is important to validate that the same conclusions can be drawn from the assessment of a plant disease outbreak using CBIIAT, regardless of the user's background and education, which was demonstrated to be true in this study. The variability among final assessment values is expected because of the differences in individual interpretations. The likelihood ranges established in the tool allow for this variability by establishing a reasonable range of point totals within which each conclusion is supported. Due to the small sample number of testers, I was unable to statistically analyze any of these results.

After the likelihood of intent is calculated for an actual investigation, the user must decide if a full-scale investigation and response are necessary. If an intentional introduction is 'likely' or 'highly likely' based on the results of the CBIIAT, the investigator should seriously consider activating the emergency response system and continue with the investigation. However, this decision should not be made solely based on the CBIIAT results; a multi-faceted approach will provide a better understanding of the response needed.

The complete tool, which can be found online at http://bioinfosu.okstate.edu/nimffab, is accompanied by a WSMV fact sheet, glossary (Appendices A and B), and example assessment. During assessment, the user is directed to consult these resources and to ask questions of the

producer and/or extension agent, if either is available. User familiarity with the resources available and understanding of the proper usage of the tool prior to assessment results in a better interpretation of the criteria, as demonstrated by Figure 2. Generally, misinterpretations of the criteria lead to deviation from the expected assessment values (Figure 5). However, the significance of this correlation could not be determined due to the low tester numbers.

Much of the information needed to complete the assessment, such as field history, human activity, possible motives and pathogen information, cannot be obtained by examining the field. Therefore, questions asked of the producer or extension agent are required for accurate value assignments. Many of the testers in this trial asked questions about every criterion, but some asked no questions at all. No relationship was identified between the number of questions asked and the deviation from the expected value (Figure 6). The most likely explanation for this is that individuals familiar with WSMV or plant pathology were able to properly assess the situation with few questions, and individuals who lacked science or WSMV experience but asked numerous questions also properly assessed the situation. Testers asked more questions during the intentional outbreak assessment than during the natural outbreak assessment, presumably due to the unusual circumstances of the former. Even though the number of questions asked did not affect the assessment outcome, the likelihood of criteria misinterpretation did appear to decrease as the number of questions rose (Figure 7), perhaps because testers obtaining more information better understood the criteria. The few exceptions to this correlation were most likely due to a misunderstanding of the criteria, a problem that was remedied by rephrasing the statements (Table 1).

Assessment of plant disease outbreaks is typically the job of a plant pathologist. However, if criteria are presented simply and resources to aid interpretation are provided, this task can be completed by investigators lacking scientific training. It is not unexpected that testers with scientific training misinterpreted the fewest criteria in this study (Figure 8). Even though the law enforcement officers investigate crimes using reasoning and logic, the investigation of a plant

disease outbreak requires specific information and an unusual investigative approach. Even though the testers' backgrounds may have influenced their ability to interpret some of the tool criteria, all of the testers in this study identified the proper intent likelihood categories, as demonstrated by the similar final point values obtained by all (Figures 3 and 8).

By combining plant disease characterization with traditional forensic investigative techniques, the possibility that a plant disease might have been incited intentionally can be assessed using the CBIIAT. Assessment is facilitated by the factsheet and example assessment that accompany the tool, as well as by recommended conversations with the producer and the extension agent responsible for the field. The utility of this tool can be enhanced if it is used in conjunction with a database containing information on reported outbreaks, where anomalies in outbreaks may be discovered. Such a database is already in place at the National Plant Diagnostic Network, a network that links plant diagnostic laboratories across the country (Stack, et al, 2006, 2007; Stack and Fletcher, 2007)

Although the CBIIAT was developed using WSMV as a model system, it can be adapted to other crop diseases by modifying the statements under each criterion. More significant alterations may be necessary to adapt the tool to diseases of plants that are not grown as annual field crops.

The CBIIAT is designed to serve as only one component in a multi-pronged investigation into the likelihood that an introduction of WSMV into a wheat field was intentional. All evidence that is collected during the investigation should be considered. The final determination of intent must be made after factoring in all aspects of the investigation.

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LITERATURE CITED

Cameron, G., and Pate, J. 2001. Covert biological weapons attacks against agricultural targets: assessing the impact against US agriculture. Terror. Polit. Violenc. 13:61-82.

Casagrande, R. 2000. Biological terrorism targeted at agriculture: the threat to US national security. The Nonproliferation Review, Fall-Winter:92-105.

Christian, M. L., and Willis, W. G. 1993. Survival of *Wheat streak mosaic virus* in grass hosts in Kansas from wheat harvest to fall wheat emergence. Plant Dis. 77:239-242.

Cupp, O. S., Walker II, D. E., and Hillison, J. 2004. Agroterrorism in the US: Key security challenge for the 21st century. Biosecur. Bioterror. 2:97-105.

Fletcher, J., Bender, C., Budowle, B., Cobb, W. T., Gold, S., Ishimaru, C., Luster, D., Melcher, U., Murch, R., Scherm, H., Seem, R.C., Sherwood, J.L., Sobral, B.W., and Tolin, S. 2006. Plant pathogen forensics: capabilities, needs, and recommendations. Microbiol. Mol. Biol. Rev. 70:450-471.

Grunow, R., and Finke, E. 2002. A procedure for differentiating between the intentional release of biological warfare agents and natural outbreaks of disease: its use in analyzing the tularemia outbreak in Kosovo in 1999 and 2000. Clin. Microbiol. Infect. 8:520-521.

Hunger, R. M., Sherwood, J. L., Evans, C. K., and Montana, J. R. 1992. Effects of planting date and inoculation date on severity of wheat streak mosaic in hard red winter wheat cultivars. Plant Dis. 76:1056-1060.

Madden, L. V., and Wheelis, M. 2003. The threat of plant pathogens as weapons againt US crops. Annu. Rev. Phytopathol. 41:155-176.

Murray, G. M., Knihinicki, D. K., Wratten, K., and Edwards, J. 2005. Wheat streak mosaic and the wheat curl mite. NSW Department of Primary Industries, Primefact 99.

National Agricultural Statistics Service. 2010. Small Grains 2010 summary. United States Department of Agriculture: Cr Pr 2-3.

National Institute of Justice. 2006. Agroterrorism-Why we're not ready: a look at the role of law enforcement. Report NCJ 214752

Niblett, C. L., Zagula, K. R., Calvert, L. A., Kendall, T. L., Stark, D. M., Smith, C. E., Beachy, R.N., and Lommel, S. A. 1991. cDNA cloning and nucleotide sequence of the *Wheat streak mosaic virus* capsid protein gene. J. Gen. Virol. 72:499-504.

OSU Plant Disease and Insect Diagnostic Laboratory. Sample submission form Retrieved December 8, 2010, from http://entoplp.okstate.edu/pddl/pdidl-form.pdf

Slykhuis, J. T. 1955. *Aceria tulipae* Keifer (Acarina: Eriophyidae) in relation to the spread of wheat streak mosaic. Phytopathology 45:116-128.

Somsen, H. W., and Sill, W. H. J. 1970. The wheat curl mite, *Aceria tulipae* Keifer, in relation to epidemiology and control of wheat streak mosaic. Kansas State University Agricultural Experiement Station, Research Publication 162.

Stack, J., Cardwell, K., Hammerschmidt, R., Byrne, J., Loria, R., Snover-Clift, K., Baldwin, W., Wisler, G., Beck, H., Bostock, R.M., Thomas, C.S., and Luke,E. 2006. The National Plant Diagnostic Network. Plant Dis. 90: 128-136.

Stack, J., and Fletcher, J. 2007. Plant biosecurity infrastructure for disease surveillance and diagnostics. *In* I. o. Medicine (Ed.), Global infectious disease surveillance and detection: Assessing the challenges - finding the solutions (Vol. Workshop summary, pp. 95-106). Washington, D.C: The National Academies Press.

Stack, J., Hammerschmidt, R., Hudler, G., Luke, E., and Bostock, R. 2007. National Plant Diagnostic Network: a record of accomplishment. CSREES five-year review. Retrieved from http://www.npdn.org/webfm_send/2

Stenger, D. C., Seifers, D. L., and French, R. 2002. Patterns of polymorphism in *Wheat streak mosaic virus*: sequence space explored by a clade of closely related viral genotypes rivals that between the most divergent strains. Virology 302:58-70.

Stromberg, E. 2003. Wheat streak mosaic, from http://www.ppws.vt.edu/stromberg/smallgrain/biology/wsmv.html

Thomas, J. A., and Hein, G. L. 2003. Influence of volunteer wheat plant condition on movement of the wheat curl mite, *Aceria tosichella,* in winter wheat. Exp. Appl. Acarol. 31:253-268.

Thomas, J. A., Hein, G. L., and Lyon, D. J. 2004. Spread of wheat curl mite and *Wheat streak mosaic virus* is influenced by volunteer wheat control methods. Plant Health Progress

Wheelis, M., Casagrande, R., and Madden, L. V. 2002. Biological attack on agriculture: low-tech, high-impact bioterrorism. BioScience 52:569-576.

CHAPTER IV

VALIDATION OF A SINGLE NUCLEOTIDE POLYMORPHISM GENOTYPING METHOD FOR *WHEAT STREAK MOSAIC VIRUS*

Abstract

The September 11, 2001 attack on the World Trade Center and the Pentagon increased the concern about the potential for terrorist attacks on many vulnerable sectors of the U.S., including agriculture. The concentrated nature of crops, easily obtainable biological agents, and highly detrimental impacts make agroterrorism a potential threat. Although procedures for an effective criminal investigation and attribution following such an attack are available, important enhancements are still needed, one of which is the capability for fine discrimination among pathogen strains. A molecular typing assay was developed, using *Wheat streak mosaic virus* (WSMV) as a model plant virus. This technique utilizes single base extension PCR to generate a genetic fingerprint composed of fifteen single nucleotide polymorphisms (SNPs) within the coat protein and helper component-protease genes. The method was validated for both detection and discrimination using nine known strains and eleven field isolates of WSMV. The assay specificity was validated using a near-neighbor panel consisting of eight genetically and environmentally similar viruses and healthy wheat tissue. The SNP fingerprints for each isolate were consistent among replicates performed by multiple investigators, demonstrating assay

reproducibility. This method incorporates molecular biology techniques that are already well established in research and diagnostic laboratories, allowing for an easy introduction of this method into existing laboratories. The specific, sensitive, and discriminatory assay will serve as a model for the development of similar tests for many other plant, animal, or human pathogens.

Introduction

The September 11, 2001 attack on the World Trade Center and the Pentagon increased the concern about terrorist attacks on many vulnerable sectors of the U.S., including agriculture (Cupp, Walker II, and Hillison, 2004). The planting density of crops, and the fact that biological agents are easily obtainable and can have highly detrimental impacts, make agroterrorism a potential threat.

A developing area of forensic science is microbial forensics, which is defined as "a scientific discipline dedicated to analyzing evidence from a bioterrorism act, biocrime, or inadvertent microorganism/toxin release for attribution purposes" (Budowle et al, 2003). As the threat of microorganisms used as weapons has become more apparent, multiple national groups are working to increase capability in this new field (Budowle et al, 2003, 2005). Enhancements of the investigative process are still being developed to close gaps that remain in key areas, such as sampling, packaging, shipping, storage, identification, and discrimination (Budowle, et al, 2003; Fletcher et al, 2006).

In addition to identifying the perpetrator of a biological crime, it is also important to identify where the pathogen originated and when and how it was introduced (Fletcher et al, 2006). Methods of plant disease diagnosis and detection are already available (Henson and French, 1993; Vincelli and Tisserat, 2008), but the stringent demands of a criminal investigation

and the pressures of the courtroom require that these methods be revised and validated for forensic use (Fletcher et al, 2006).

A single nucleotide polymorphism (SNP) method for molecular typing of *Wheat streak mosaic virus* (WSMV) was adapted recently from protocols designed originally to test human DNA in forensic applications (Bouakaze et al, 2007; Carver, 2007; Grignani et al, 2006; Kohnemann et al, 2008; Quintans et al, 2004). By comparing three strains of WSMV, Carver (2007) identified three SNPs in the coat protein (CP) region. SNP fingerprint was generated using the PRISM® SNaPshot™ Multiplex Kit (Applied Biosystems, Inc., Foster City, CA). Each strain had a unique pattern of electropherogram peaks, demonstrating the discriminatory power of this new method (Carver, 2007). The SNaPshot multiplex kit also has been successfully used for serotype grouping of *Potato virus* Y isolates (Rolland et al, 2008).

Molecular comparisons using SNPs are often used for evolutionary, ecological, and forensic studies (Bouakaze et al, 2007; Bujalkova et al, 2008; Grignani et al, 2006; Jungerius et al, 2003; Kawuki et al, 2009; Kohnemann et al, 2008; Quintans et al, 2004; Ranade et al, 2001; Rolland et al, 2008), and can be especially helpful in typing organisms with high mutation rates, such as RNA viruses with mutations caused by an error-prone RNA-dependent RNA polymerase (French and Stenger, 2005). The CP gene of WSMV is the most variable region of the genome with several conserved regions corresponding to the functionally important residues (Shukla et al, 1991). Other genes with highly variable regions include helper-component protease (HC-Pro), protein 1 (P1), and protein 3 (P3) (Choi et al, 2001; Shukla et al, 1991). The high mutation rate characteristic of RNA viruses contributes to the evolution of this virus and to its existence as a quasi-species, having multiple variants in a single population. Using SNPs, analysis of these variants can discriminate among populations, making this a powerful tool that provides benefits that techniques such as sequencing, which focus on identifying the consensus of the population, do not offer.

WSMV, an important pathogen of wheat in the United States (U.S.), was selected as the model pathosystem for the development of this molecular typing method. WSMV belongs to the genus *Tritimovirus* within the family *Potyviridae* (Stenger et al, 2002). The virus genome is 9384 nucleotides (nt) long and is translated as a single polyprotein processed by three viral proteinases (Stenger, et al, 2002). WSMV has a positive sense, single-stranded RNA genome (Niblett et al, 1991). This feature is common to most plant viruses, which allows this new SNP typing method to be applied almost universally, with minor modifications, to most plant viruses. Wheat streak mosaic (WSM) is a relatively common disease every year and is found in most of the wheat growing regions of the U.S. The high prevalence of the virus provides access to many field isolates across the nation. Previous studies have focused on genetic diversity and evolution, including mutation rates, phylogenetic positions and polymorphic sites (Choi et al, 2001; French and Stenger, 2003, 2005; Hall et al, 2001, 2001; Rabenstein et al, 2002; Stenger et al, 2002). Excellent detection methods using PCR have also already been established (Berger and Shiel, 1998; Chen et al, 2001; French and Robertson, 1994). Extensive knowledge of the viral genome and the availability of robust detection assays facilitate the development of a more stringent, discriminatory assay for variant characterization.

In this work, I enhanced the discriminatory power of the WSMV molecular typing method by increasing the number of targeted SNPs. I expanded the assay to include the HC-Pro gene, a highly variable gene of WSMV having multiple functions including gene-silencing, vector transmission, and proteolytic cleavage (Stenger and French, 2004; Stenger et al, 2005, 2005, 2006; Stenger, Young, and French, 2006; Young et al, 2007). I validated the method by determining the sensitivity, specificity and reproducibility of the assay, as well as its ability to discriminate among known strains of WSMV and several field isolates.

Materials and Methods

I - Selection of virus strains

WSMV strains were received from virus collections at Oklahoma State University in Stillwater, OK, the University of Nebraska in Lincoln, NE, and from AGWEST Plant Laboratories in South Perth, West Australia. Other infected wheat samples were collected in Oklahoma wheat fields and from the Great Plains Diagnostic Network (GPDN) wheat virus survey of 2008. *Oat necrotic mottle virus* (ONMV), *Wheat spindle streak mosaic virus* (WSSMV), and *Maize dwarf mosaic virus* (MDMV) strain A were received from a virus collection at Oklahoma State University. High plains virus (HPV), *Barley yellow dwarf virus* PAV (BYDV-PAV), and *Cereal yellow dwarf virus* RPV (CYDV-RPV) were received from the GPDN wheat virus survey. All samples were maintained in dry, frozen leaf material at -80°C, with the exception of the Australian WSMV isolate Ginnindera, which was stored at -80°C as an RNA pellet.

II - SNP identification

SNPs were identified by comparing the sequences of 85 different WSMV sequences obtained from the GenBank database, including the strains Sidney 81 (Accession: AF057533.1), Type (Accession: AF285169.1), and El Batan 3 (Accession: AF285170.1). The CP and the HC-Pro segments of the genome were targeted for the SNP-typing method because of the high variability among strains (Choi et al, 2001; Shukla et al, 1991). The polymorphic sites selected contained high variability among the strains and were surrounded by semi-conserved regions that would provide a sufficient template for primer binding.

III - Nucleic acid extraction

Total RNA was extracted from healthy or infected *Triticum aestivum* leaves using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD) according to manufacturer's instructions. A total of 100 mg of tissue was homogenized at full speed for 90 seconds using a mini bead-beater (Biospec Products, Bartlesville, OK) and 550 µl of the RLT lysis buffer. The RNA was eluted using 50µl of RNase-free water supplied with the kit and the concentration determined using the Nanodrop v.2000 (Thermo Scientific, Waltham, MA).

IV - Positive control

A synthetic plasmid derived from the cloning vector pUC57 was created for each of the target regions of WSMV (produced by GenScript, Piscataway, NJ). The CP synthetic plasmid contains nt 8061-9428 and the HC-Pro synthetic plasmid contains nt 1081-2507 (based on Sidney 81 genome from GenBank, Accession #AF057533). The plasmids were used during the one-step reverse transcription-PCR (RT-PCR) at a concentration of 2 ng/µl and were processed through the entire method as a sample.

V - cDNA synthesis and amplification

cDNA was synthesized and amplified from total RNA in a single step using the SuperScript™ III One-Step RT-PCR kit with Platinum© *Taq* (Invitrogen, Carlsbad, CA), following the protocol supplied by the manufacturer. In separate reactions primer C1 (5'-TACTTGACTGGGACCCGAA-3'; Sidney 81 nt 8117 to 8134) was used for the CP reverse-transcription and primer HCR (5'- CATGCTTGTATACTGAGAACAGTCTCTTG-3'; Sidney 81 nt 2373 to 2345) was used for the HC-Pro reverse-transcription to synthesize the DNA strand complementary to the RNA sequence (Chenault et al, 1996; French and Stenger, 2005; McNeil et al, 1996). The CP region of the cDNA was amplified using the sense primer C1 and the anti-sense primer XC1 (5'-AACCCACACATAGCTACCAAG-3'; Sidney 81 nt 9371 to 9351) (Carver, 2007; Chenault et al, 1996; French and Stenger, 2005; McNeil et al, 1996). The HC-Pro region

of the cDNA was amplified using the anti-sense primer HCR and the sense primer HCF (5'-GAAATGCACACATGGACTTAGATGGTAT-3'; Sidney 81 nt 1159 to 1186) (Stenger and French, 2004). These primers produce a ~1.3 kb amplicon for both CP and HCPro that contains the gene and flanking regions (based on the Sidney 81 genome from GenBank, Accession: AF057533).

Each RT-PCR reaction contained the following components: 25 µl 2X reaction mix (supplied with kit), 1 µg template RNA (6 ng of synthetic plasmid), 0.2 µM of each primer (C1 and XC1, or HCR and HCF), 2.0 mM $MgSO_4$, 1µl of RT/Platinum *Taq* mix (supplied with kit), and sterile water up to 50 µl. The kit protocol consisted of cDNA synthesis (one cycle of 50°C for 60 minutes and 94°C for 2 minutes), amplification (35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 1 minute) and elongation (68°C for 5 minutes).

The amplification of the CP and HCPro genes was verified by electrophoresis using a 1.5% agarose gel in 1X TAE buffer containing SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, CA). The samples and a 100 bp DNA ladder (Invitrogen, Carlsbad, CA) were loaded with 1X loading dye containing bromophenol blue and xylene cyanol and were electrophoresed for 30 minutes at 100 mV. The gel was visualized and photographed using ultraviolet radiation.

VI - Post-amplification treatment

To prevent any further binding of the primers or dNTPs, the samples were purified using shrimp alkaline phosphatase (SAP) and Exonuclease 1 (Exo1) (Affymetrix, Inc., Cleveland, OH). A mixture containing 1 unit of SAP, 2 units of Exo1, and sterile water up to 16 µl was added to the 50 µl PCR product. The mixture was incubated at 37°C for 30 minutes followed by 75°C for 15 minutes to inactivate the enzymes. The sample was used immediately in further experiments or stored at -20°C.

VII - SNaPshot technology

I used a PRISM® SNaPshot™ Multiplex Kit (Applied Biosystems, Foster City, CA), which provides a mechanism of SNP identification by utilizing only fluorescently labeled dideoxynucleotides (ddNTPs) during the elongation of template-specific primers during PCR. The chemical composition of ddNTPs prevents any further elongation, resulting in a PCR product that is one nucleotide longer than the primer itself. Methods were those of the manufacturer.

Specific WSMV primers were designed for each targeted SNP (Table 1). Each primer was of a different length, ranging from 30-65 nt, to facilitate distinguishing the products during analysis. The primers bound to the DNA template within one nucleotide downstream of the polymorphic site. During the elongation step, a fluorescent-ddNTP complementary to the polymorphic nucleotide was added and elongation was terminated. Included in each assay were controls that were supplied with the SNaPshot kit to verify the performance of the PCR components.

Each reaction contained 5 μl SNaPshot Multiplex Ready Reaction Mix (provided with kit), 3 μl purified PCR product, 1 μl of pooled PCR primers (final concentration of 0.2 μM each), and 1 μl of sterile deionized water. The products were amplified using the following two-step program: 25 cycles of 96°C for 10 seconds and 60°C for 35 seconds. After amplification was complete, the samples were treated with SAP to prevent further binding of ddNTPs. One unit of SAP, 2 μl of 10X SAP buffer, and 7 μl of nuclease-free water was added to each SNaPshot reaction. The mixtures were incubated for 30 minutes at 37°C followed by 15 minute incubation at 75°C.

Table 1: SNaPshot primers designed for WSMV

Name[a]	Length (nt)	Sequence (5'-3')[b]	T_M	Strand[c]	Final Conc. (uM)
30C	30	GACTGGCGTGTTCTCCCTCACATCATCTGC	65.10	F	0.1
35C	35	GACTGACTGACAACTGAACAACTCAACACCTGGAT	63.50	R	0.2
40C	40	GACTGACTGACTGACTGGCATATCTGTTGTCGATAAGTTC	63.50	F	0.1
45C	45	GACTGACTGACTGACTGACTGTAGTTTCTACTGTGCTCACGCAAG	66.50	F	0.2
50C	50	GACTGACTGACTGACTGACTGACTTTCGCAGCTTTGTACATCGGTTCAAT	67.60	F	0.4
55C	55	GACTGACTGACTGACTGACTGACTGACTGATCTCTGGGCACGTTGTGTTGATTAT	72.05	F	0.4
60C	60	GACTGACTGACTGACTGACTGACTGACTGACTGACCCCTTGGTATGATAGGTTTTCCAAT	72.82	F	0.4
65C	65	GACTGACTGACTGACTGACTGACTGACTGACTGACTGACTTGTTCTGTATTCCGCGTAGCCTGTT	74.74	F	0.4
30H	30	GACTGCAAGTTGTCTTAGCACATCACCAAA	60.80	F	0.4
35H	35	GACTGACTGACTGACTGCGCAAGTTACCTGGAAGC	66.30	R	0.4
40H	40	GACTGACTGACTGACTGCGTATCCAGACAGCGATGTAACA	66.40	R	0.4
45H	45	GACTGACTGACTGACTGACTGAATTGTTCCATCTTCTAGCATCTT	64.70	F	0.1
50H	50	GACTGACTGACTGACTGACTGACTGACTCGAATTTTAGGTTCGTTGATTT	65.80	R	0.1
55H	55	GACTGACTGACTGACTGACTGACTGACTGACTTTAGACTCGCCGTTAATTTCCAT	71.31	F	0.4
60H	60	GACTGACTGACTGACTGACTGACTGACTGACTGACTGATTTGTCACTGGGAAGGAAATGT	72.82	R	0.2

[a] The letter of the primer name indicates the target is either the coat protein (C) or the helper-component protease (H) segment of the genome.
[b] Each primer contains a segment that anneals to the template (underlined) and a GACT tail to adjust the length of the primer
[c] The primer anneals to either the forward (F) or reverse (R) strand.

The purified SNaPshot products were separated by capillary electrophoresis on an ABI 3730 Genetic Analyzer after combining 0.5 µl purified SNaPshot product, 0.5 µl GeneScan 120-LIZ size standard, and 9 µl Hi-Di Formamide (Applied Biosystems, Foster City, CA). The parameters suggested in the SNaPshot protocol were used. The separated products were analyzed using Peak Scanner Software v1.1 (Applied Biosystems, Foster City, CA).

VIII- Data analysis

Multiple datasets were created from the SNP profiles for quantitative analyses, including data from each peak having a peak height greater than 800 FUs (this limit was set based on background noise, explained below). The first two datasets were created using peak height values; one composed of the absolute fluorescence values after normalization based on positive control values; the second composed of percent of each nucleotide in each SNP position. Two additional data sets were created similarly, but based on the area under the curve instead of peak height. Both peak height and area under the curve were included in the analyses to determine which fluorescent value was more reliable for profile comparisons. Each dataset was statistically analyzed using analysis of variance (ANOVA), assuming a randomized complete block model and a p-value <0.05. The strain and the SNP position were the random blocking effects and the replicate was the function of interest.

Using the percent nucleotide dataset from the area under the fluorescent peak values, a principle component analysis (PCA) was performed using Canoco software (Biometris-Plant Research International, Wageningen, The Netherlands) with square-root transformation. The WSMV strains and field isolates were included in the analysis, in addition to the *in silico* SNP profile data for strains El Batan 3 and Czech to provide a known dimension of diversity.

Results

I - Generation of SNP-specific primers

One hundred and twenty-two SNPs within the CP of WSMV were previously identified (Stenger et al, 2002). Ten of these SNPs were selected for inclusion in this assay because of the high variability between each strain and the presence of a semi-conserved region surrounding the SNP that provides a sufficient template for primer binding. The remaining CP SNPs for this assay were identified by multiple sequence alignment of 85 different WSMV sequences obtained from GenBank, including the strains Sidney 81, Type, and El Batan 3. The SNPs in the HCPro region of the genome were identified by performing a multiple sequence alignment of 13 WSMV HCPro sequences obtained from the NCBI database. The same criteria were used for selection of the SNPs. Due to the limited number of SNPs having an adjacent template sufficient for primer binding, both the positive and negative sense strands of the viral template were used.

A 20-25nt hybridizing region was designed for each SNP with the addition of a $[GACT]_n$ tail to the 5'-end to provide a unique length for each primer, ranging from 30-65nt. Using *Oligocalc* software *(*Kibbe, 2007*)*, the thermodynamics and folding structures were checked for each primer. Each primer was checked for specificity to WSMV using BLASTn with the NCBI database. Any primer that was not specific to WSMV or possessed folding structures with a ΔG greater than 3.0°C was eliminated from the study.

The remaining SNP-specific primers were screened for inclusion in the assay by performing monoplex and multiplex *in vitro* tests using synthetic targets that were specific to each primer. Primers generating a single fluorescent peak in the SNaPshot electropherogram and no peaks in the negative control were selected for inclusion in the WSMV SNaPshot assay (Fig.1). These primers are listed in Table 1.

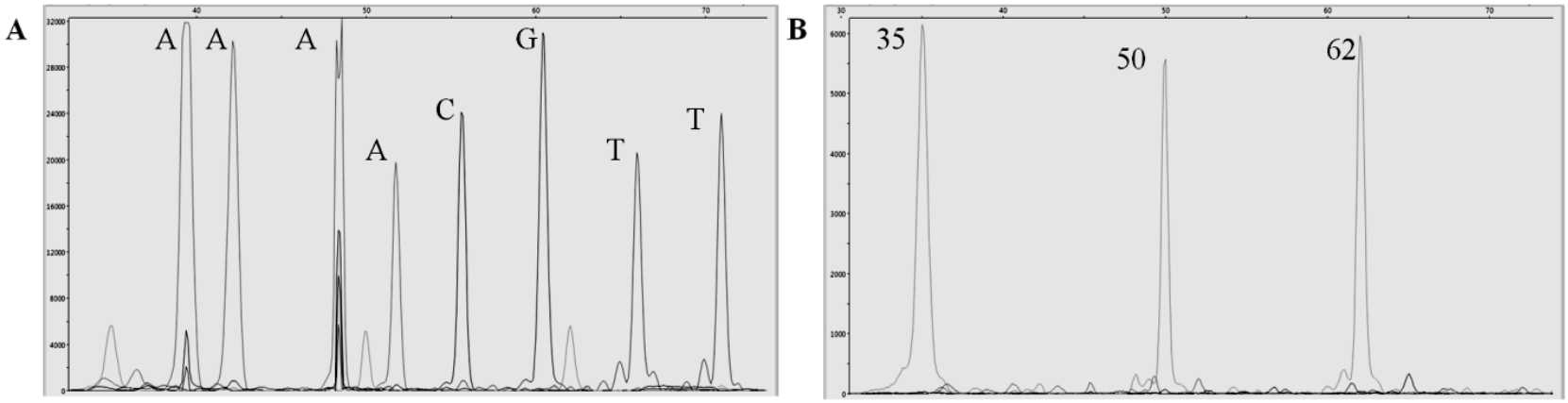

Figure 1: Black and White image of CP multiplex electropherogram. The height of each peak indicates the concentration of fluorescent units and the location of the peaks along the x-axis indicates the size of the fragment in nucleotides; left to right showing smallest to largest. The color of each peak (labeled here according to nucleotide) indicates the nucleotide being attached (green, red, yellow, and blue represent an A, T, C, and G, respectively). A) Amplification of synthetic targets using eight SNP-specific primers for the WSMV CP, showing a single amplified target for each primer. B) Negative control for the CP amplification, using sterile deionized water instead of the synthetic templates, which demonstrates the absence of non-target amplification. The peaks present (labeled according to fragment size) are size standards to help with the orientation of the fragment sizes.

II - Sensitivity and limit of detection

The sensitivities of the CP and HCPro assays were determined using synthetic plasmids containing the target region. A 1:3 dilution series was created from a starting solution of 0.075 pmole/µl. The SNaPshot reaction was performed and the electropherogram generated by capillary electrophoresis. There was a linear relationship between the fluorescent units and the amount of DNA (pmoles) in each sample (Fig. 2).

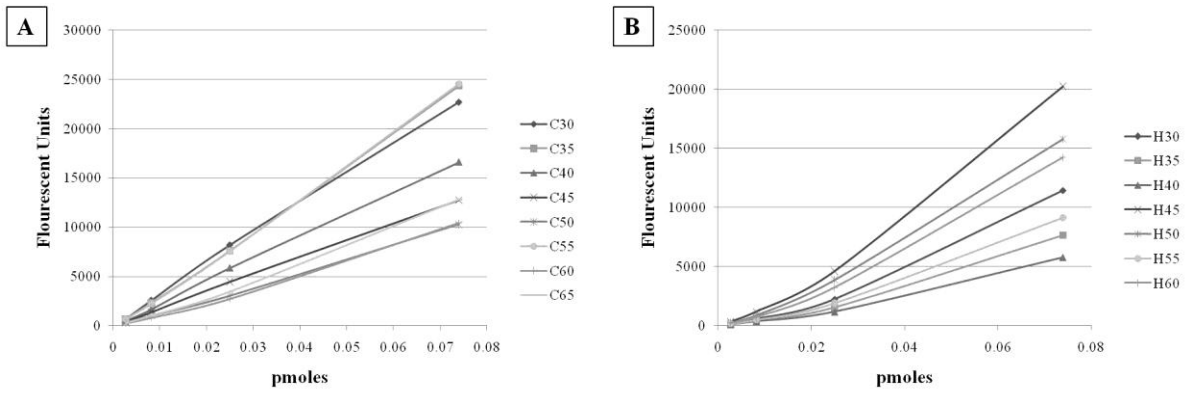

Figure 2: The amount of fluorescence generated by number of pmoles added to the CP (A) and the HCPro (B) SNaPshot assays. Both graphs demonstrate a linear relationship for each SNP (individual lines). The sensitivity of the assay was determined by identifying the pmoles that correspond to 800FU using the linear equation of the SNP with the steepest slope (C55 and H45). Linear equations: y=336285x-475.19 and y=285041x-1239, CP and HCPro, respectively.

The background noise in the electropherograms of the negative and positive controls consistently appeared between 300 and 750 fluorescent units (FUs) (data not shown). To reduce the possibilities of false positives from background peaks, the limit of detection was set at 800 FUs, and this level was applied to determine the sensitivity of detection by calculating the number of pmoles that corresponds to 800 FUs; 0.00379 pmoles and 0.00715 pmoles for CP and HCPro, respectively.

III - Specificity to WSMV

Genetic and environmental near-neighbors to WSMV were tested for RT-PCR amplification and SNaPshot assay detection. These viruses included ONMV, WSSMV, MDMV, HPV, BYDV-PAV and CYDV-RPV, and healthy *Tricitum aestivum* cv. Chisholm. Figure 3 shows the gel electrophoresis results, demonstrating the lack of the WSMV characteristic ~1300 bp amplicon in the near-neighbor samples and the presence of this amplicon in the WSMV positive controls. Amplicons were present in three samples from the CP assay: HPV, CYDV-RPV, and BYDV-PAV. *In silico* analysis using NCBI BLASTn demonstrates that portions of the C1 and XC1 primers are complementary to regions within each of their genomes. However, the primer sequence is not 100% complementary to any of the three, which may explain the presence of the faint bands. These three samples were subjected to the SNaPshot assay and no fluorescent peaks were present in electropherograms, demonstrating that the SNaPshot assay is specific to WSMV.

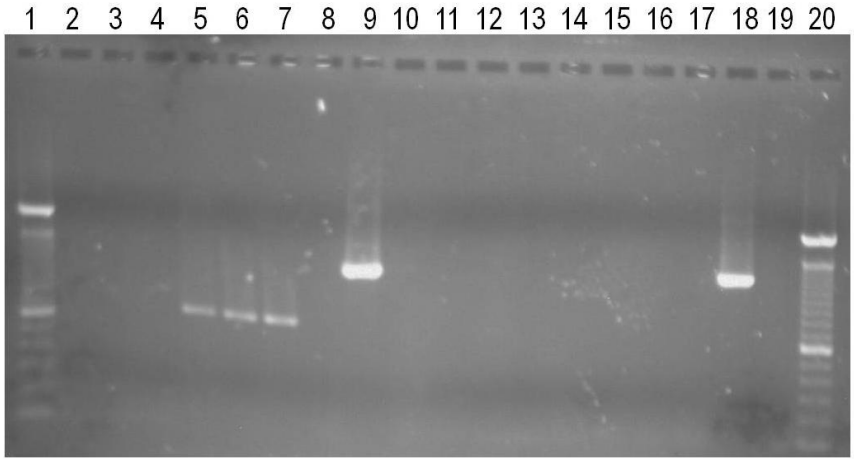

Figure 3: Gel electrophoresis of CP and HCPro amplification of WSMV near-neighbors. Lanes 2-8 and 11-17 are ONMV, WSSMV, MDMV, HPV, CYDV-RPV, BYDV-PAV, and healthy wheat PCR products for CP and HCPro assays, respectively. Lanes 1 and 20 are a 100bp DNA ladder. Lanes 9 and 18 are the positive control CP and HC-PRO WSMV plasmid, respectively. Lanes 10 and 19 are negative controls. Only the positive controls produced ~1300 bp amplicons, corresponding to WSMV.

IV - Characterization of WSMV strains and field isolates

The discriminatory ability of the SNaPshot assay was tested using known strains of WSMV (Sidney 81, type, OSU, Alvaro, Franco, and Ginnindera) and 16 field isolates. A PCR amplicon of the appropriate size for the CP and HCPro was obtained for each sample (Fig. 3). Each strain and most field isolates produced a unique SNaPshot fingerprint (Table 2). However, some field isolates that came from the same geographical region had nearly identical fingerprints, such as three samples from Montana (Conrad 1, Kalispell, and Huntley B).

Table 2: SNaPshot fingerprints of WMSV strains and field isolates using the IUB nucleotide code±.

Sample	Origin	SNP Profile	
		HC-Pro	CP
Sidney 81	Nebraska	M C C A G C G A W R R Y G Y W	
Type	Kansas	K Y Y A G M G A A C A T G C C	
OSU	Oklahoma	B Y Y A G B G A A C A Y G Y W	
Alvaro	Australia	K C C A G C G A W S R T G C W	
Franco	Australia	K Y Y A G S G A A C A T G C W	
Ginnindera	Australia	K Y Y A G V G A A C A T G T T	
Billings	Montana	T C C A G C G A A A A Y R Y Y	
Conrad1	Montana	C C C A G C G A A A A C A T T	
Conrad2	Montana	T M T A G C G R A A M Y R Y Y	
Conrad3	Montana	T C T A A C G R A C A T G C C	
Helena	Montana	C C C A G C G A A M A Y G Y T	
HuntleyA	Montana	Y C C A G C G A A A A Y A Y Y	
HuntleyB	Montana	C C C A G C G A A A A C A T T	
Kalispell	Montana	C C C A G C G A A A A C A T T	
Toole	Montana	T C C A G C G A A - A T G C C	
OK-KF	Oklahoma	K C Y A G S G A A S R C G Y W	
OK-CC	Oklahoma	Y C C A G C G A A A A C G T T	
OK-Lab	Oklahoma	B C Y A G C G A A M A Y G B Y	
OK-425	Oklahoma	K C C A G C G W A V R H S B W	
CO-17	Colorado	K C C R G C G A A M A Y G Y H	
NE-932	Nebraska	K C S A G C G A A V R T G C H	
KS-117	Kansas	K C Y A G S G A A S R C G Y W	
*El Batan 3	Mexico	A C C A C T C A C G A C G C C	
*Czech	Czech	T A T G A G T G C T C C G T C	

* indicates *in silico* results based on the sequence obtained from GenBank.

± IUB nucleotide code: R=AG, Y=TC, K=TG, M=AC, S=GC, W=AT, B=CGT, D=AGT, H=ACT, V=ACG, N=AGCT (NC-IUB, 1986)

The relationship among these samples was depicted using PCA with the inclusion of the *in silico* SNaPshot results of El Batan 3 and Czech WSMV strains (Fig. 4). The strains Czech, El Batan 3, Sidney 81, and Toole provide a dimension of diversity as these strains have been thoroughly tested in previous phylogenetic studies (French and Stenger, 2005; Hall et al, 2001; Rabenstein et al, 2002; Stenger et al, 1998, 2002). The genetic variability among these samples as depicted in the PCA is consistent with the current understanding of WSMV genetic diversity. The known strains and the field isolates show a high level of diversity with no apparent segregation of field isolates based on geographic location. The results indicate that these samples, even those obtained from Australia, are more closely related to Sidney 81 and Type than El Batan 3 or Czech, which is consistent with previous analyses of the U.S. WSMV population (Choi et al, 2001; French and Stenger, 2003, 2005; Rabenstein, et al, 2002).

The original assay developed by Carver (2007) utilized four SNPs, three of which remain in the assay described here. If only the initial three were used to discriminate among these samples (40 nt, 50 nt, and 55 nt CP primers), only 14 genotypes (58%) would have been identified compared to the 21 (87%) identified using 15 SNPs. This demonstrates an enhanced discriminatory power with an increased number of targets.

V - Reproducibility of assay

The SNaPshot profiles were generated three times from cDNA of the six WSMV strains to test for the reproducibility of results. Each replicate produced identical SNaPshot fingerprints for each sample based on qualitative results (data not shown). The SNaPshot profile was generated once more from cDNA of the same samples by another operator and the fact that the results of the second operator were nearly identical to those of the first (Table 3) demonstrate that the results are not noticeably altered by their performance by another operator. The discrepancies among the profiles generated from the two operators are due to differences in fluorescent signals

because different amounts of cDNA were mistakenly used. However, all the fluorescent peaks were visible in all replicates, but the pre-determined limit of detection eliminated some of the minor peaks from inclusion in base-calling. To determine if the results (fluorescent units) were quantitatively reproducible, a statistical analysis using ANOVA was performed. During the analysis, each nucleotide was compared within each SNP position for each sample replicate. The results for each nucleotide were then compiled into a single number to represent the level of variance. Looking at the results of the peak height-absolute fluorescence data set analysis, all four nucleotides were statistically significant (Table 4). However, only nucleotides G and T were found to be statistically significant when the analysis was performed on the peak height-nucleotide percent data set. The same results were found for both of the area under the curve data sets with the exception of nucleotide G in the area under the curve – nucleotide percent data. The statistical significance indicates the fluorescence values are different among each strain replicate, however, the difference is minimal when using the area under the curve dataset.

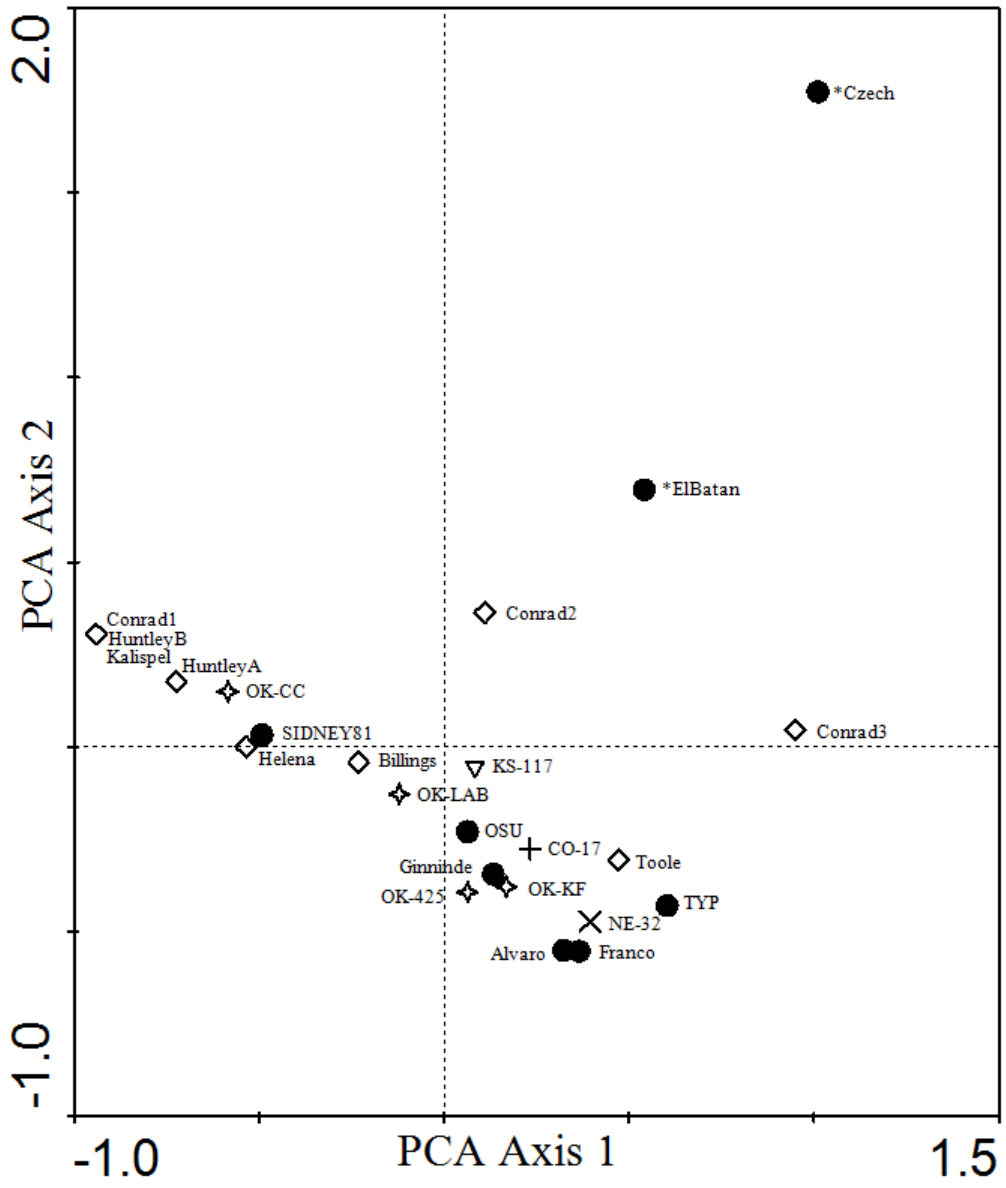

Figure 4: Principle component analysis of WSMV strains and field isolates. The known strains are depicted by solid circles and the field isolates by the remaining symbols (open diamond=Montana, open star=Oklahoma, cross=Colorado, filled diamond=Kansas, X=Nebraska). The majority of the samples form a weak cluster between the Sidney 81 and Type strains, with El Batan 3 and Czech strains appearing as outliers. *= profiles generated *in silico* using GenBank sequence.

Table 3: WSMV strain SNP fingerprint similarity obtained by two different operators.

Strain	Percent Identity*
OSU	92% (22/24)
Sidney 81	86% (19/22)
Type	84% (16/19)
Alvaro	85% (17/20)
Franco	85% (17/20)
Ginnindera	95% (20/21)

*Identity refers to an exact match between nucleotides.

Table 4: ANOVA results of WSMV strain replicates

Dataset	Effect	Nucleotide	Num DF*	Den DF+	F Value	Pr > F±
Peak height – percent						
	Replicate	A	2	178	0.35	0.7075
	Replicate	C	2	178	0.64	0.5266
	Replicate	G	2	178	4.88	0.0087
	Replicate	T	2	178	3.44	0.0343
Peak height – absolute						
	Replicate	A	2	178	13.22	<0.0001
	Replicate	C	2	178	4.05	0.0190
	Replicate	G	2	178	17.73	<0.0001
	Replicate	T	2	178	11.72	<0.0001
Area under curve - percent						
	Replicate	A	2	178	0.96	0.3845
	Replicate	C	2	178	1.46	0.2342
	Replicate	G	2	178	0.75	0.4757
	Replicate	T	2	178	3.73	0.0259
Area under curve -absolute						
	Replicate	A	2	178	7.57	0.0007
	Replicate	C	2	178	10.30	<0.0001
	Replicate	G	2	178	8.21	0.0004
	Replicate	T	2	178	3.35	0.0375

*Num DF= Numerator Degrees of Freedom
+Den DF= Denominator Degrees of Freedom
±results are significant if Pr value is less than 0.05

Discussion

The ability to discriminate among viral isolates can be useful in evolutionary and ecological studies and for forensic investigations in which determining the origin of a biological

agent is critical to criminal attribution. For example, identifying the source of the pathogen became a crucial aspect of the investigation of the anthrax letter attacks in 2001, but because appropriate scientific tools for strain discrimination were not available at that time it was not until seven years later that a suitable test was developed and validated, ultimately leading to attribution (Budowle et al, 2005). The development and validation of universal, robust, sensitive, and specific detection and discriminatory assays is important for a rapid and efficient response to biological attacks that may occur on our agricultural industry or nation.

The assay described here is intended for use in a forensic investigation, but it can be useful also for other molecular studies in which viral variant discrimination may be necessary. Increasing the number of SNPs targeted in the assay from three to fifteen significantly enhances the discriminatory power. The relationship between SNP number and discrimination ability is comparable to that of human DNA fingerprinting, in which the use of as many as thirteen alleles results in an extremely low probability that a genetic fingerprint could be found in any other individual based on allele frequencies in the population (Budowle et al, 2001). Because none of the probes failed to reveal a variation, each SNP is informative and should be included in the assay. Although I did not test strain El Batan 3 or any of the European strains *in vitro*, their theoretical profiles were included in the data analysis to demonstrate the estimated discriminatory ability of the method. The fifteen SNPs I used for WSMV provide a high level of confidence that the fingerprint is unique to the variant population without testing a prohibitive number of SNPs.

In my laboratory, the SNP method discriminated among several known strains of the virus and also among field isolates collected throughout the United States and in Australia. Several of the profiles are similar but have minor differences in the secondary peaks that appear for some of the SNPs. These secondary peaks are due to the presence of multiple variants in the population, a feature that adds a higher level of discrimination than can be achieved through consensus sequence analysis. Many of the primary peaks are similar among the strains and

isolates because most of the strains and isolates belong to the same clade of WSMV (Stenger et al, 2002).

In my hands, the limit of detection for the CP assay and HC-Pro assay, respectively, were 3.79 fmoles and 7.15 fmoles of WSMV cDNA, both of which are lower than the detection limit of 10 fmoles reported in the SNaPshot Kit manual. The level of sensitivity of this assay is comparable to that of other SNP-based techniques, RT-PCR, and real-time PCR (Henson and French, 1993).

In applications of the SNaPshot assay to field samples it is important to consider that many organisms and viruses other than the target may be encountered, and that some of these, such as Triticum mosaic virus (TriMV) and HPV, which are commonly found in the same infected tissue (Brakke, 1987; Jensen et al, 1996; Tatineni et al, 2009), may be genetically related to WSMV. I tested many of the viruses known to co-infect wheat with WSMV. However, since TriMV is almost always found with WSMV (Burrows et al, 2009), I was unable to obtain TriMV infected wheat tissue that was not also infected with WSMV; therefore, TriMV was not included in the specificity study. However, the primers used in this assay were confirmed to be WSMV specific through *in silico* analyses. Our *in vitro* specificity testing demonstrated that the genetically similar viruses and ecologically similar organisms included in this study would neither interfere with the assay nor provide false positives. This was confirmed by the lack of fluorescent signal during capillary electrophoresis of the SNaPshot products for HPV, CYDV-RPV, and BYDV-PAV.

For the successful implementation of this assay in a forensic investigation, the SNP profile of a single sample must be reproducible by the same operator and also other operators, which was demonstrated in this study for qualitative comparisons. The inability to utilize this assay in a quantitative capacity does not hamper its utility in forensic investigations, as the

presence/absence of SNPs in a profile should provide sufficient information. However, quantitative analyses may be beneficial for epidemiological or ecological studies where detecting the amount of genetic change may provide insight into organismal interactions or disease development.

This specific, sensitive, and discriminatory SNaPshot assay has been developed using WSMV as a model system. Similar assays could be developed for application to many other plant, animal, or human pathogens by designing organism-specific primers, a task that is becoming ever more feasible with the increasing availability of microbial sequence data and primer designing software. This method already has been shown to be useful for other pathogens, such as *Potato virus* Y (Rolland et al, 2008), and for genetic comparisons of humans and other organisms (Bouakaze et al, 2007; Bujalkova et al, 2008; Ceriotti et al, 2004; Civaova and Knoll, 2007; Grignani et al, 2006; Kawuki et al, 2009; Kohnemann et al, 2008; Murphy et al, 2007; Ovesna et al, 2003; Quintans et al, 2004). The assay uses molecular biology techniques that are well established already in many research and diagnostic laboratories, allowing for a seamless introduction of the technology into existing laboratories.

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LITERATURE CITED

Berger, P. H., and Shiel, P. J. 1998. Potyvirus isolation and RNA extraction. Method Mol. Biol. 81:151-160.

Bouakaze, C., Keyser, C., Amory, S., Crubezy, E., and Ludes, B. 2007. First successful assay of Y-SNP typing by SNaPshot minisequencing on ancient DNA. Int. J. Legal. Med. 121:493-499.

Brakke, M. 1987. Virus disease in wheat. In E. Heyne (Ed.), Wheat and Wheat Improvement 2nd Edition, pp. 585-603. Madison, WI: American Society of Agronomy, Crop Science Society of America, Soil Science Society of America.

Budowle, B., Burans, J. P., Breeze, R., Wilson, M., and Chakraborty, R. 2005. Microbial Forensics. In R. Breeze, B. Budowle and S. E. Schutzer (Eds.), Microbial Forensics (pp. 1-26). San Diego, CA: Elsevier Academic Press.

Budowle, B., Johnson, M. D., Fraser, C. M., Leighton, T. J., Murch, R. S., and Chakraborty, R. 2005. Genetic analysis and attribution of microbial forensics evidence. Crit. Rev. Microbiol. 31:233-254.

Budowle, B., Schutzer, S. E., Einseln, A., kelley, L., Walsh, A. C., Smith, J., Marrone, Babetta L., Robertson, J., and Campos, J.2003. Public health: Building microbial forensics as a response to bioterrorism. Science 301:1852-1853.

Budowle, B., Shea, B., Niezgoda, S., and Chakraborty, R. 2001. CODIS STR loci data from 41 sample populations. J. Forensic Sci. 46:453-489.

Bujalkova, M., Zavodna, K., Krivulcik, T., Ilencikova, D., Wolf, B., Kovac, M., karner-Hanusch, J., Heinimann, K., Marra, G., jiricny, J., and Bartosova, Z. 2008. Multiplex SNaPshot

genotyping for detecting loss of heterozygosity in the mismatch-repair genes *MLH1* and *MSH2* in microsatellite-unstable tumors. Clin. Chem. 54:1844-1854.

Burrows, M. E., Franc, G., Rush, C. M., Blunt, T., Ito, D., Kinzer, K., Olson, J., O'Mara, J., Price, J. A., Tande, C., Ziems, A., and Stack, J. 2009. Occurence of viruses in wheat in the Great Plains region, 2008. Online. Plant Health Progress. doi:10:1094/PHP-1009-0706-01-RS.

Carver, J. 2007. Molecular typing of *Wheat streak mosaic virus* for forensic applications. M.S., Oklahoma State Univeristy-Center for Health Sciences, Tulsa, OK.

Ceriotti, G., Chessa, S., Bolla, P., Budelli, E., Bianchi, L., Duranti, E., and Caroli, A. 2004. Single nucleotide polymorphisms in the ovine casein genes detected by polymerase chain reaction-single stranded conformation polymorphism. J. Dairy Sci. 87:2606-2613.

Chen, J., Chen, J., and Adams, M. 2001. A universal PCR primer to detect members of the *Potyviridae* and its use to examine the taxonomic status of several members of the family. Arch. Virol. 146:757-766.

Chenault, K. D., Hunger, R. M., and Sherwood, J. L. 1996. Comparison of the nucleotide sequences of the coat protein open reading frame of nine isolates of *Wheat streak mosaic rymovirus*. Virus Genes 13:187-188.

Choi, I.-R., Hall, J. S., Henry, M., Zhang, L., Hein, G. L., French, R., and Stenger, D. C. 2001. Contributions of genetic drift and negative selection on the evolution of three strains of *Wheat streak mosaic tritimovirus*. Arch. Virol. 146:619-628.

Civaova, K., and Knoll, A. 2007. SNaPshot minisequencing and a panel of candidate genes for complex routine testing of meat performance traits in pigs. Anim. Biotechnol. 18:109-115.

Cupp, O. S., Walker II, D. E., and Hillison, J. 2004. Agroterrorism in the US: Key security challenge for the 21st century. Biosecur. Bioterror. 2:97-105.

Fletcher, J., Bender, C., Budowle, B., Cobb, W. T., Gold, S., Ishimaru, C., Luster, D., Melcher, U., Murch, R., Scherm, H., Seem, R.C., Sherwood, J.L., Sobral, B.W., and Tolin, S.2006. Plant pathogen forensics: capabilities, needs, and recommendations. Microbiol. Mol. Biol. Rev. 70:450-471.

French, R., and Robertson, N. L. 1994. Simplified sample preparation for detection of *Wheat streak mosaic virus* and *Barley yellow dwarf virus* by PCR. J. Virol. Methods 49:93-100.

French, R., and Stenger, D. C. 2003. Evolution of *Wheat streak mosaic virus*: Dynamics of population growth within plants may explain limited variation. Annu. Rev. Phytopathol. 41:199-214.

French, R., and Stenger, D. C. 2005. Population structure within lineages of *Wheat streak mosaic virus* derived from a common founding event exhibits stachastic variation inconsistent with the deterministic quasi-species model. Virology 343:179-189.

Grignani, P., Peloso, G., Achilli, A., Turchi, C., Tagliabracci, A., Alu, M., Beduschi, G., Ricci, U., Giunti, L., Robino, C., Gino, S., and Previdere, C. 2006. Subtyping mtDNA haplogroup H by SNaPshot minisequencing and its application in forensic individual identification. Int. J. Legal Med. 120:151-156.

Hall, J. S., French, R., Hein, G. L., Morris, T. J., and Stenger, D. C. 2001. Three distinct mechanisms facilitate genetic isolation of sympatric *Wheat streak mosaic virus* lineages. Virology 282:230-236.

Hall, J. S., French, R., morris, T. J., and Stenger, D. C. 2001. Structure and temporal dynamics of populations within *Wheat streak mosaic virus* isolates. J. Virol. 75:10231-10243.

Henson, J. M., and French, R. 1993. The polymerase chain reaction and plant disease diagnosis. Ann. Rev. Phytopath. 31:81-109.

Jensen, S. G., Lane, L. C., and Seifers, D. L. 1996. A new disease of maize and wheat in the high plains. Plant Dis. 80:1387-1390.

Jungerius, B. J., Veenendaal, A. T., van Oost, B. A., te Pas, M. F., and Groenen, M. A. 2003. Typing single-nucleotide polymorphisms using a gel-based sequencer. Mol. Biotechnol. 25:283-287.

Kawuki, R. S., Ferguson, M., Labuschagne, M., Herselman, L., and Kim, D.-J. 2009. Identification, characterisation and application of single nucleotide polymorphisms for diversity assessment in cassava (*Manihot esculenta* Crantz). Mol. Breeding, 23:669-684.

Kibbe, W. A. 2007. OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res. 35:W43-W46.

Kohnemann, S., Sibbing, U., Pfeiffer, H., and Hohoff, C. 2008. A rapid mtDNA assay of 22 SNPs in one multiplex reaction increases the power of forensic testing in European Caucasians. Int. J. Legal Med. 122:517-523.

McNeil, J. E., French, R., Hein, G. L., Baenziger, P. S., and Eskridge, K. M. 1996. Characterization of genetic variability among natural populations of *Wheat streak mosaic virus.* Phytopathology 86:1222-1227.

Murphy, S. J., hughes, A. E., Patterson, C. C., Anderson, L. A., Watson, R. G. P., Johnston, B. T., Comber, H., McGuigan, J., Reynolds, J.V., and Murray, L. J. 2007. A population-based assocation study of SNPs of *GSTP1, MnSOD, GPX2* and Barrett's esophagus and esophageal adenocarcinoma. Carcinogenesis 28:1323-1328.

Niblett, C. L., K. R. Zagula, L. A. Calvert, T. L. Kendall, D. M. Stark, C. E. Smith, R. N. Beachy, and S. A. Lommel 1991. cDNA cloning and nucleotide sequence of the *Wheat streak mosaic virus* capsid protein gene. J. Gen. Virol. 72:499-504.

Nomenclature Committee of the International Union of Biochemistry (NC-IUB). 1986. Nomenclature for incompletely specified bases in nucleic acid sequences: recommendations 1984. PNAS 83:4-8.

Ovesna, J., Polakova, K., Kucera, L., and Rulcova, J. 2003. SNP typing in cereals: comparison of SSCP and SNaPshot markers using Barley *Mlo* locus as a model. Czech J. Genet. Plant Breed. 39:109-112.

Quintans, B., Alvarez-Iglesias, V., Salas, A., Phillips, C., Lareu, M., and Carracedo, A. 2004. Typing of mitochondrial DNA coding region SNPs of forensic and anthropological interest using SNaPshot minisequencing. Forensic Sci. Int. 140:251-257.

Rabenstein, F., Seifers, D. L., Schubert, J., French, R., and Stenger, D. C. 2002. Phylogenetic relationships, strain diversity, and biogeography of tritimoviruses. J. Gen. Virol. 83:895-906.

Ranade K., Chang M.-S., Ting C.-T., Pei D., Hsiao C.-F., Olivier M., Pesich R., Hebert J., Chen Y.-D.I., Dzau V.J., Curb D., Olshen R., Risch N., Cox D.R., and Botstein D. 2001. High-throughput genotyping with single nucleotide polymorphisms. Genome Res. 11:1262-1268.

Rolland, M., Glais, L., Kerlan, C., and Jacquot, E. 2008. A multiple single nucleotide polymorphisms interrogation assay for reliable *Potato virus* Y group and variant characterization. J. Virol. Methods 147:108-117.

Shukla, D., Frenkel, M., and Ward, C. 1991. Structure and function of the potyvirus genome with special reference to the coat protein coding region. Can. J. Plant Pathol. 13: 178-191.

Stenger, D. C., and French, R. 2004. Functional replacement of *Wheat streak mosaic virus* HC-Pro with the corresponding cistron from a diverse array of viruses in the family *Potyviridae*. Virology 323:257-267.

Stenger, D. C., French, R., and Gildow, F. E. 2005. Complete deletion of *Wheat streak mosaic virus* HC-Pro: a null mutant is viable for systemic infection. J. Virol. 79:12077-12080.

Stenger, D. C., Hall, J. S., Choi, I.-R., and French, R. 1998. Phylogenetic relationships within the family *Potyviridae*: *Wheat streak mosaic virus* and *Brome streak mosaic virus* are not members of the genus *Rymovirus*. Phytopathology 88:782-787.

Stenger, D. C., Hein, G. L., and French, R. 2006. Nested deletion analysis of *Wheat streak mosaic virus* HC-Pro: Mapping of domains affecting polyprotein processing and eriophid mite transmission. Virology 350:465-474.

Stenger, D. C., Hein, G. L., Gildow, F. E., Horken, K. M., and French, R. 2005. Plant virus HC-Pro is a determinant of eriophyid mite transmission. J. Virol, 79:9054-9061.

Stenger, D. C., Seifers, D. L., and French, R. 2002. Patterns of polymorphism in *Wheat streak mosaic virus*: sequence space explored by a clade of closely related viral genotypes rivals that between the most divergent strains. Virology 302:58-70.

Stenger, D. C., Young, B. A., and French, R. 2006. Random mutagenesis of wheat streak mosaic virus HC-Pro: non-infectious interfering mutations in a gene dispensable for systemic infection of plants. J. Gen. Virol. 87:2741-2747.

Tatineni, S., Ziems, A. D., Wegulo, S. N., and French, R. 2009. *Triticum mosaic virus*: A distinct member of the Family *Potyviridae* with an unusually long leader sequence. Phytopathology 99:943-950.

Vincelli, P., and Tisserat, N. 2008. Nucleic acid-based pathogen detection in applied plant pathology. Plant Dis. 92:660-669.

Young, B. A., Hein, G. L., French, R., and Stenger, D. C. 2007. Substitution of conserved cysteine residues in *Wheat streak mosaic virus* HC-Pro abolished virus transmission by the wheat curl mite. Arch. Virol. 152:2107-2111.

CHAPTER V

DETERMINING THE EFFECT OF FIELD TREATMENTS ON THE GENETIC PROFILE OF *WHEAT STREAK MOSAIC VIRUS*

Abstract

Plant pathogens of wheat are often controlled using chemical compounds. In addition to the use of antimicrobials, pesticides may be used to control arthropod vectors, and fertilizer is commonly applied to both enhance grain yield and render the crop less susceptible to pathogen attack. These compounds, in addition to environmental conditions, may alter the plant's physiology, change the habitat of resident microorganisms, and support the sustainability of mutated pathogen genotypes. Changes in the genotype content and ratios within a pathogen population over time could impair effective forensic analysis. Using *Wheat streak mosaic virus* (WSMV) as a model system, I evaluated the effect of several field treatments and conditions on the genetic profile of the virus over two growing seasons. Quantitative and qualitative comparisons of WSMV SNP profiles were used to create a phylogenetic tree using maximum likelihood software. Assessment of relatedness using principle component analysis revealed variations among the samples, but there was no correlation between field treatment and pathogen profiles. The variation observed is consistent with those found in previous studies on WSMV genetic diversity within the United States, suggesting the variation is a natural phenomenon. While these field treatments and conditions do not affect the genotype ratios, the

variation observed should be considered when developing strategic sampling and data assessment methods for forensic investigations.

Introduction

Recently, the emerging threat of bioterrorism and the vulnerabilities present within the U.S. agricultural industry have led to a blending of the fields of plant pathology and forensic science (Budowle et al, 2003, 2005a, 2005b, 2005c, 2005d, 2006; Cummings and Relman, 2002). The practice of planting a single crop variety, the increasing size of U.S. farms, and the natural abundance of easily obtainable plant pathogens heighten the risk of a covert attack on U.S. cropping systems (Cupp et al, 2004; Fletcher et al, 2006). Criminal investigation of such an event would involve intersection of the law enforcement and security communities with the agricultural community; a setting that is rarely encountered in a forensic investigation. To increase the likelihood of criminal attribution in such cases, many national groups are working to develop effective protocols for pathogen detection and discrimination and for sample collection and processing in agricultural investigations (Budowle et al, 2003, 2005, 2005; Fletcher et al, 2006). While many methods for plant pathogen detection and identification exist already, stringent assay validation is required for use in a criminal case (Budowle et al, 2006; Fletcher et al, 2006). Furthermore, forensically valid plant and environmental sampling techniques are needed.

The U.S. is the world's largest exporter of wheat, a staple crop worldwide, (US Wheat Associates, 2010). Every year, wheat production is challenged by fungal, bacterial and viral pathogens. Common control methods include fungicide applications for the control of many fungal diseases, insecticide applications for the control of arthropods and aphids that are common vectors of viral pathogens, and cultural practices such as destruction of volunteer wheat and weeds and adjustment of planting date. Annual or bi-annual applications of fertilizer are commonly made to increase grain yields and support robust growth, thereby minimizing plant

susceptibility to pathogen attack. These amendments may alter the physiology of the plant, creating a different environment for the resident microorganisms (Blandino and Reyneri, 2009; Grossmann and Retzlaff, 1997; Krieg et al, 2003; Molyneux and McKinlay, 1989; Walker et al, 2009) and leading to the survival of newly emerging pathogen genotypes.

Wheat streak mosaic virus (WSMV), a common pathogen of wheat in the U.S. and elsewhere, is a single-stranded positive sense RNA virus belonging to the Family *Potyviridae* and genus *Tritimovirus* (Stenger et al, 1998). Primarily due to its error-prone RNA polymerase, WSMV has a high mutation frequency of ~5.0 x 10^{-4}/nt; however, genetic and physical bottlenecks encountered during systemic movement in the host plant prevent most of these mutants and other genetic variants from moving into other cells (French and Stenger, 2003, 2005). Virus sequence diversity from field to field within a single geographic area is as great as that among several geographic regions (Choi et al, 2001; Hall et al, 2001). Hall et al (2001) identified a number of singleton substitutions in a WSMV population, suggesting that genetic drift plays a major role in the variations observed. Choi et al (2001) showed a similar result with negative selection also contributing to the variation. Using WSMV as a model pathosystem, I developed and validated an assay to generate a genetic profile of the virus population utilizing single nucleotide polymorphisms (SNPs) (Chapter 2).

During a forensic investigation, elucidation of the original source of the virus will require effective field sampling and generation of virus genetic profiles. Sampling options include random, targeted, or statistically derived approaches (Budowle et al, 2006). Due to the possibility of changes in the physiology of the wheat plant after chemical applications, it is important to understand how such changes may affect the genotype of the viral population.

In this study, I inoculated a wheat field with WSMV, applied different agronomic treatments to different field sections, and assessed changes in the WSMV SNP profile over time

in each section. The treatments, which included fertilization, fungicide or insecticide application, or shading, were designed to mimic conditions likely to be encountered during an investigation of a WSMV outbreak. The findings of this study may assist in the development of sampling strategies that will appropriately represent the genetic diversity of plant pathogens in a field setting and ultimately contribute to the identification of the introduction source.

Materials and Methods

I - Selection of virus strain

WSMV infected leaf tissue was obtained from our collection at Oklahoma State University (OSU). This laboratory maintained isolate of WSMV (referred to as Lab08 and Lab09 for the 2008 and 2009 field studies, respectively) was initially collected from an infected wheat field in Oklahoma in 2004. The SNP profile of the isolated virus indicated that, as is normal in natural WSMV outbreaks, multiple viral variants were present. WSMV strains Sidney 81 and Type were obtained from Roy French (University of Nebraska – Lincoln) as infected wheat leaf tissue and stored at -80°C.

II - Field inoculation

Winter wheat, *Triticum aestivum* cv. 'Vona', was planted at the OSU research farm in Stillwater, OK, using a Hege drill set on #24 for 27 kg per 4046.872 610 m² in November and October of 2008 and 2009, respectively. Approximately one month prior to planting, 46-0-0 fertilizer (Agri-Nutrients, Catoosa, OK) was applied at a rate of 81 kg of material (37 kg of actual N) per 4046.872 610 m². The laboratory-maintained isolate of WSMV was spray inoculated onto month old winter wheat as described by Hunger et al (1992). The 361 m^2 field was divided into eight, 21 m^2 plots, each containing seven, 1.1 m^2 subplots with 1.2 m wide borders of wheat surrounding each subplot (Figure 1). Five of the seven subplots were sprayed with the WSMV

inoculum containing Celite® as a wounding agent and the remaining two were sprayed with buffer (distilled water with Celite®).

III - Treatments

Each plot received one of several agronomic treatments commonly applied by wheat producers or designed to mimic natural environmental conditions. Durable nylon sunshades that were designed to block approximately 75% of the light were placed approximately 1.2 m above the ground over one plot immediately following inoculation to mimic shade provided by overhanging trees or a cloudy season. Fertilizer (46-0-0), in the form of urea pellets (Agri-Nutrients, Catoosa, OK), was applied to one plot in late February at a rate of 15 kg per 4046.872 610 m². Fertilizer is typically applied shortly before a rain event to ensure the movement of nitrogen into the soil. Due to drought conditions in both study years, I simulated a 6.4 cm rain event by sprinkling 2.4 cm^3 of water onto each subplot following fertilizer application. Spectracide Malathion insecticide (Spectrum Brands, Madison, WI) was applied to one plot every two weeks after aphids were first observed (mid March) until harvest by spraying the emulsified concentrate at a rate of 2.3 cm^3 per 4046.872 610 m² with a handheld sprayer. An application at Feekes stage 10 (Bockus, 2010) of Stratego® fungicide (Bayer, Morrisville, NC) was applied on two plots using a handheld sprayer at a rate of 10 fl oz per 4046.872 610 m², with one plot also receiving an early application (stage 8 wheat). The remaining plots were controls and received no treatment. Care was taken to ensure treatments were limited to the intended plot by performing spray applications only when wind speeds were lower than 5 mph and by including at least 1.2 m-wide strips of wheat between each plot to serve as a buffer.

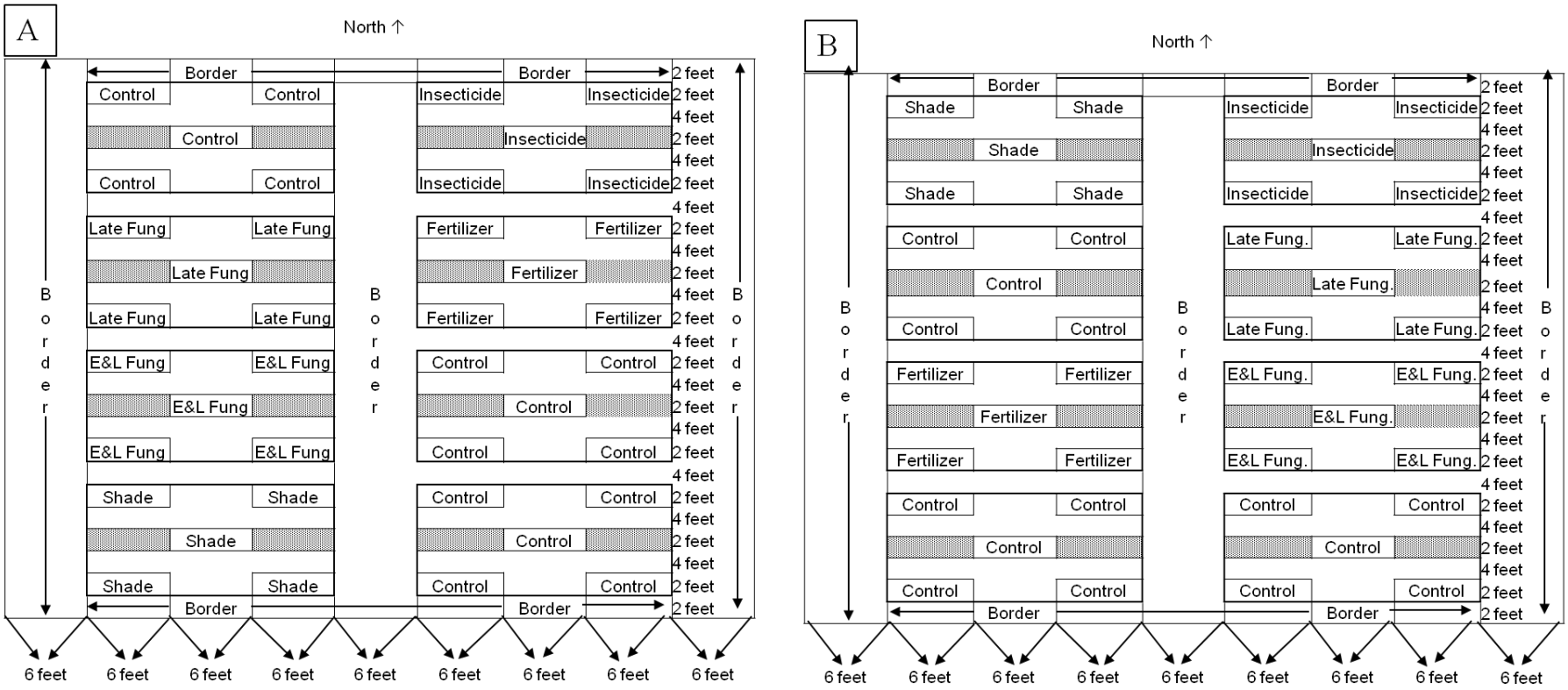

Figure 1: Field study plot design for (A) 2008-2009 growing season and (B) 2009-2010 growing season. The shaded boxes indicate the buffer control subplots and the labeled boxes are the WSMV inoculated subplots indicating treatment. Borders are rows of wheat to serve as barriers between treatments.

IV - Sample collection

Three leaves (symptomatic when applicable) were collected randomly from the western-most, center, and eastern-most portions of each subplot immediately before inoculation, two weeks following inoculation, and every two months throughout the growing season, for a total of five time points. Each sample was labeled with an identification code indicating the time of collection, year of study, and plot and subplot identification. After collection, the leaf samples were pooled for each subplot and stored at -80°C until sample processing.

V - Nucleic acid extraction

Total RNA was extracted from leaf tissue using the RNeasy Plant Mini Extraction Kit (Qiagen, Germantown, MD). The pooled samples for each subplot were diced (~25 mm^2) and thoroughly mixed before weighing out 100 mg. After adding 550 µl RLT buffer, the samples were homogenized at full speed for 90 seconds using a Mini-BeadBeater (Biospec Products, Bartlesville, OK). RNA was eluted from the spin columns using 50 µl RNase-free water, supplied with the kit.

VI - RT-PCR

The coat protein (CP) and helper component-protease (HC-Pro) genes of WSMV were synthesized and amplified using a SuperScript III One-Step RT-PCR Kit with Platinum *Taq* (Invitrogen, Carlsbad, CA) following manufacturer's instructions. The RT-PCR procedure and post-extension treatment is presented on page 79 in Chapter IV. Product amplification was confirmed through electrophoresis of 5 µl samples using a 1.5% agarose gel containing SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA) and 1X TAE buffer. A 100 bp DNA ladder (Invitrogen, Carlsbad, CA) was used to estimate product size. The gels were electrophoresed for 30 min at 100 mV/cm and products visualized and photographed using ultraviolet radiation.

VII - Single base extension PCR

SNP fingerprints were generated for each confirmed WSMV positive sample using the PRISM SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA). As described on page 80 in Chapter IV, a single fluorescently-labeled ddNTP was incorporated into the position complementary to the target SNP. After post-extension treatment with shrimp alkaline phosphatase (Affymetrix, Inc., Cleveland, OH), the products were separated using an ABI 3730 Genetic Analyzer and the resulting electropherograms visualized using Peak Scanner Software v1.1 (Applied Biosystems, Foster City, CA).

VIII - Data analysis

For each SNP position, fluorescent peak higher than 800 FU were used in the analysis. SNP profile sequences were recorded for each sample using the IUB nucleotide code (NC-IUB, 1986) to account for heterogeneity in the SNPs. A separate phylogenetic analysis was performed for each field trial, generating a phylogenetic tree using the maximum likelihood (ML) algorithm and Tamura-Nei model followed by a bootstrap with 1000 replicates. The trees were rooted at the starting inoculum (Lab08 and Lab09 for the 2008 and 2009 field plots, respectively). Nucleotide changes were traced over the bootstrap consensus ML tree using the MEGA 5.0 software (Tamura et al, 2011).

The numbers of heterogeneous SNP positions in each sample are shown in Tables 3 and 4. The field treatment corresponding to each heterogeneous position was examined to identify any correlation between treatment and heterogeneity. The SNP positions were also evaluated for heterogeneity by calculating the number of samples that were heterogeneous at each position (Tables 5 and 6).

The primary peak profile was generated for each sample by selecting the peak having the highest fluorescent area value in each SNP position and compiling a sequence that represents the

predominant viral variant in each sample. WebLogo 3.0 software (Crooks et al, 2004) was used to generate a graph of the differences among the aligned sequences.

The nucleotide percentages in every SNP position were calculated for each sample using Microsoft Excel. The dataset was analyzed using analysis of variance (ANOVA), assuming a randomized complete block model and a p-value <0.05. The year of study and the SNP position were the random blocking effects and the treatment was the function of interest. The relationships among the samples within the respective field trial were evaluated by principle component analysis (PCA) on square-root transformed data using Canoco software (ter Braak and Smilauer, 2002). The SNP profiles generated for the three best characterized WSMV strains (Sidney 81, Type, and El Batan 3) were included in the nucleotide percentage data set to provide a dimension of known genetic diversity. The El Batan 3 SNP profile was generated *in silico* because a biological sample of the virus was not available to us (NCBI Accession # AF285170.1).

Results

I - Generation of SNP profile sequences

SNP profile sequences were derived for February and May samples from the 2008 field trial and for April samples from the 2009 field trial. As illustrated in Tables 1 and 2, most of the plots had at least three positive samples, ranging from 0-4 positives. No positives were obtained from the May 2008 fertilizer plot or the 2008 shade plot. For 2009, only the April samples were included in the analysis due to the low number of positive samples obtained at the other 2009 sampling dates. WSMV was not detected in the negative control subplots, suggesting that the WSMV infection present in the treated plots resulted from the intentional introduction (*data not shown)*.

Table 1: WSMV SNP profiles of 2008 field samples using the IUB nucleotide code±.

Treatment	Sample[a]	SNP Profile Sequence
Starting Inoculum	LAB08	B C Y A G C G A A M A Y G B Y
Early & Late Fungicide	F8B1	K C C A G C G A A C A T G T M
	F8B3	T C C A G C G A A C A T G C W
	F8B4	K C Y A G C G A A C A T G Y T
	M8B2	C C C A G M G A A C A C G G C
	M8B5	C C C A A C G A A A A C G T T
Late Fungicide	F8C1	B C C A G C G A A C A Y G K T
	F8C2	B C C A G C G A A C A Y G B W
	F8C5	K Y T A G V G A A C A C G T W
	M8C2	K C C A G C G A A C A T G Y T
	M8C3	Y C C A A C G A A A A C G T W
Control	F8D2	H C C A G C G A A A A Y G T W
	F8D3	T C C A G C G A A C A T G C W
	F8D4	T C C A G C G A A C A T G C W
	M8D1	Y C C A G C G A A C A T G C W
	M8D2	T C C A G C G A A C A T G C W
	M8D4	K Y C A G V G A A C A T G Y W
Fertilizer	F8G2	K C C A G C G A A C A T G C W
	F8G3	K C T A G S G A A C A T G C T
	F8G4	T C C G G C G A A C A T G C T
Insecticide	F8H1	K C C A G C G A A A A T G C C
	F8H3	K C Y A G C G A A C A T G C W
	M8H1	K C C A G C G A A C A T G T W
	M8H2	C C C A G C G A A A A C G T T
	M8H3	K C C A G C G A A T A T G C W

[a] Sample ID: month (F=February, M=May), year (8=2008), plot (B,C,D,G,H), subplot (1-5)
± IUB nucleotide code: R=AG, Y=TC, K=TG, M=AC, S=GC, W=AT, B=CGT, D=AGT, H=ACT, V=ACG, N=AGCT (NC-IUB, 1986)

Table 2: WSMV SNP profiles of 2009 field samples using the IUB nucleotide code±.

Treatment	Sample[a]	SNP Profile Sequence
Starting Inoculum	LAB09	B C C A G S G A A M A Y G B H
Early & Late Fungicide	A9F1	C C C A A C G A A A A C G Y W
	A9F3	Y C C A G C G A A C A Y G Y H
	A9F5	K C C V G S G A A C R T G T W
Late Fungicide	A9G1	C C C A G M G A A C A C G T T
	A9G4	K C C A G C G A A C A T G C W
	A9G5	T C C A G C G A A C A T G T W
Control	A9C3	K C C A G C G A A C R C G C W
	A9C4	Y C C A R C G A A A A Y G Y W
	A9C5	C C C A A M G A A A R C G T W
Fertilizer	A9B1	T C Y A G C G M A S R W G C W
	A9B3	Y C C A G C G A A C A T G T C
Insecticide	A9H5	K C C A G C G A A C A T G C W
Shade	A9D5	T C T A G C G W A C R T G C W

[a] Sample ID: month (A=April), year (9=2009), plot (B,C,D,F,G,H), subplot (1-5)
± IUB nucleotide code: R=AG, Y=TC, K=TG, M=AC, S=GC, W=AT, B=CGT, D=AGT, H=ACT, V=ACG, N=AGCT (NC-IUB, 1986)

The SNP profiles (Tables 1 and 2) were similar regardless of treatment, but a few heterogeneous positions were present through the growing season. A graphical representation of the primary profile of each sample was generated using WebLogo 3.0 (Figure 2). Seven of the fifteen SNP positions show a mixture of nucleotides. However, the major nucleotide in each position is consistent with the primary profile of the starting inoculum, which suggests that the predominant starting viral variant was maintained throughout the season. Almost identical results were obtained in the 2009 field trial (Figure 2B).

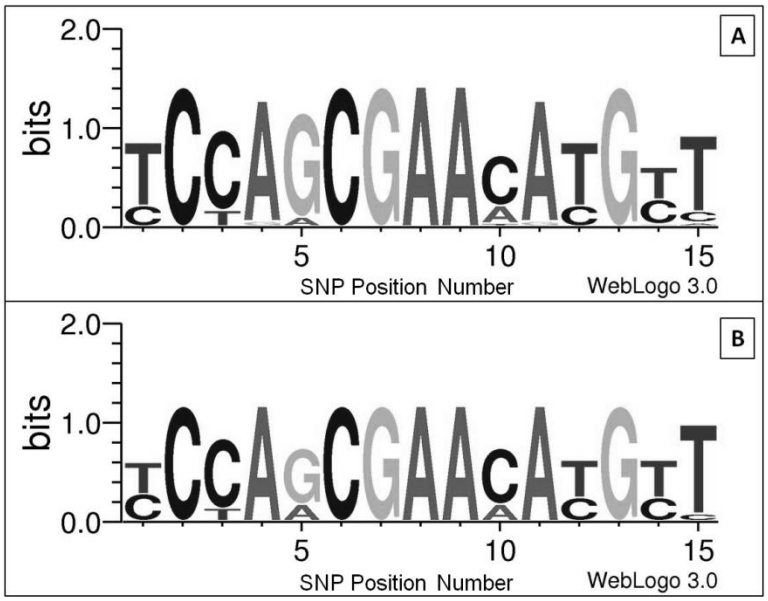

Figure 2: Alignment of the primary nucleotide in each SNP position for the (A) 2008 and (B) 2009 WSMV field samples. The size of the nucleotide (bits) indicates the conservation of the nucleotide in the alignment. The SNP position number correlates with the HCPro gene (30-60 nt fragment) and CP gene (30-65nt fragment). Starting inoculum primary profile: TCCAGCGAACATGTT

While several SNP positions in the multi-variant profiles were heterogeneous, several others were well-conserved. SNP positions 2, 4, 5, 7, 8, 9, 11, and 13 are highly conserved in both field trials (Tables 3 and 4). SNP positions 1 and 15 had the greatest heterogeneity in both the 2008 and 2009 trials (Tables 5 and 6). The starting inoculum had six heterogeneous positions, but most of the samples contained fewer than six. No correlation was found between number of heterogeneous positions and field treatment. However, the mean number of heterogeneous positions decreased for each treatment except the control plot from February to May 2008. The overall means for February and May also show a slight decrease in number of heterogeneous positions, but with no other month sampling for the 2009 study, this result cannot be confirmed. The mean values calculated in 2009 are higher than those found in 2008, with the late growing season insecticide treatment samples having the second to lowest mean in both years.

Table 3: Number of heterogeneous 2008 WSMV field samples in each queried SNP position out of 24 samples.

SNP position	Heterogeneous samples/position	Percentage of total samples
1	16	67
2	2	8
3	2	8
4	0	0
5	0	0
6	4	16
7	0	0
8	0	0
9	0	0
10	0	0
11	0	0
12	3	13
13	0	0
14	5	21
15	15	63

Table 4: Number of heterogeneous 2009 WSMV field samples in each queried SNP position out of 13 samples.

SNP position	Heterogeneous samples/position	Percentage of total samples
1	7	54
2	0	0
3	1	8
4	1	8
5	1	8
6	3	23
7	0	0
8	2	15
9	0	0
10	1	8
11	5	38
12	3	23
13	0	0
14	3	23
15	11	85

Table 5: Number and identity of heterogeneous SNP positions for each 2008 WSMV field sample.

Treatment	Sample[a]	Number of Heterogeneous Positions	Treatment Mean	Month Mean	Positions[b]
N/A	Lab08	6			1,3,10,12,14,15
Early & late fungicide	F8B1	2	2.0	2.1	1,15
	F8B3	1			15
	F8B4	3			1,3,14
Late fungicide	F8C1	3	3.7		1,12,14
	F8C2	4			1,12,14, 15
	F8C5	4			1,2,6,15
Control	F8D2	3	1.6		1,12,15
	F8D3	1			15
	F8D4	1			15
Fertilizer	F8G2	2	1.3		1,15
	F8G3	2			1,6
	F8G4	0			
Insecticide	F8H1	1	2.0		1
	F8H3	3			1,3,15
Early & late fungicide	M8B2	1	0.5	1.6	6
	M8B5	0			
Late fungicide	M8C2	2	2.0		1,14
	M8C3	2			1,15
Control	M8D1	2	2.7		1,15
	M8D2	1			15
	M8D4	5			1,2,6,14,15
Insecticide	M8H1	2	1.3		1,15
	M8H2	0			
	M8H3	2			1,15

[a] Sample ID: month (F=February, M=May), year (8=2008), plot (B,C,D,G,H), subplot (1-5)
[b] SNP position number indicates HCPro gene (1-7) and CP gene (8-15)

Table 6: Number and identity of heterogeneous SNP positions for each 2009 WSMV field sample.

Treatment	Sample[a]	Number of Heterogeneous Positions	Treatment Mean	Positions[b]
N/A	Lab09	6		1,6,10,12,14,15
Fertilizer	A9B1	6		3,8,10,11,12,15
	A9B3	1	3.5	1
Control	A9C3	3		1,11,15
	A9C4	5		1,5,12,14,15
Shade	A9C5	3	3.6	6,11,15
	A9D5	3	3.0	8,11,15
Early & late fungicide	A9F1	2		14,15
	A9F3	4		1,12,14,15
	A9F5	5	3.6	1,4,6,11,15
Late fungicide	A9G1	1		6
	A9G4	2		1,15
	A9G5	1	1.3	15
Insecticide	A9H5	2	2.0	1,15

[a] Sample ID: month (A=April), year (9=2009), plot (B,C,D,F,G,H), subplot (1-5)
[b] SNP position number indicates HCPro gene (1-7) and CP gene (8-15)

II - Phylogenetic analysis

ML trees for the 2008 and 2009 field trials (Figures 3 and 4) depict the relationships among the field samples. In 2008, the samples clustered within their respective treatments, but the bootstrap values are low, providing little support for a true phylogenetic basis for the tree topology or for correlation with plot treatment. The 2009 data (Figure 4) fail to show a relationship between position and treatment, with low bootstrap values, with the exception of the node containing an early and late fungicide sample and a late fungicide sample (A9F3 and A9G1), which has a bootstrap value of 85. Again, the character tracing fails to demonstrate a pattern correlated with treatment. In both years, the control samples are dispersed among the treated field samples, suggesting that the genetic variation may be a natural phenomenon.

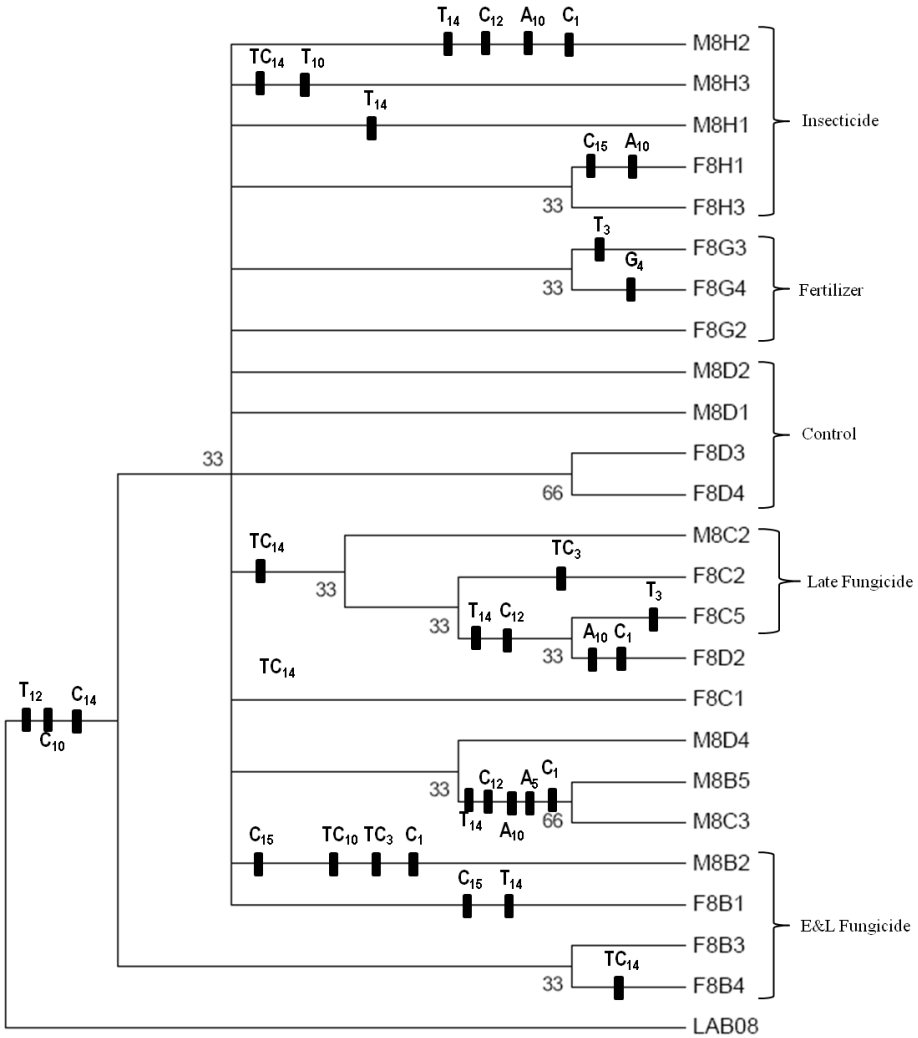

Figure 3: Maximum Likelihood tree with character tracing of WSMV SNP profiles for the 2008 field trial, rooted to the starting inoculum (LAB08). The samples are denoted by month (F=February, M=May), year (8=2008), treatment (B=early and late fungicide, C=late fungicide, D=control, G=fertilizer, H=insecticide), and subplot (1-5). All nucleotide changes (major and minor) for each SNP position are traced on the tree, showing the nucleotide with a subscript indicating the SNP position (HC-Pro:1-7; CP:8-15). Bootstrap values indicated at applicable nodes.

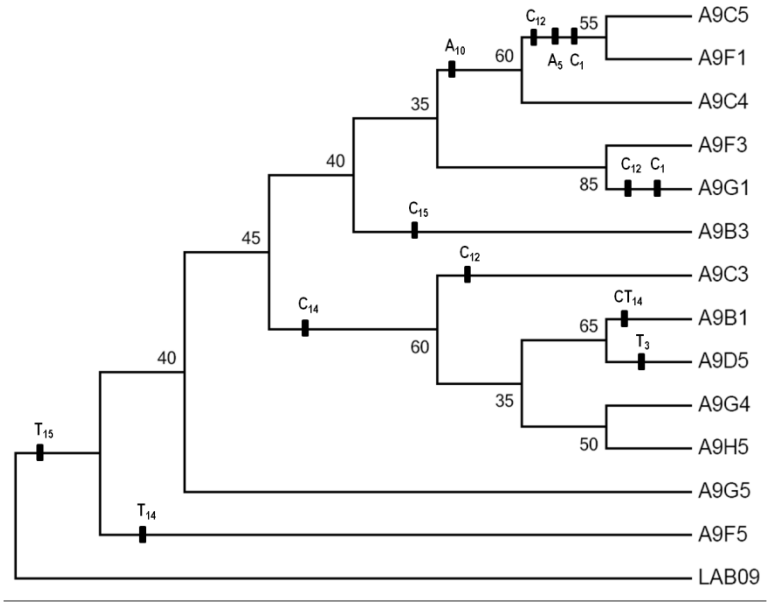

Figure 4: Maximum Likelihood tree with character tracing of WSMV SNP profiles for the 2009 field trial, rooted to the starting inoculum (LAB09). The samples are denoted by month (A=April), year (9=2009), treatment (B=fertilizer, C=control, D=shade, F=early and late fungicide, G=late fungicide, H=insecticide), and subplot (1-5). All nucleotide changes (major and minor) for each SNP position are traced on the tree, showing the nucleotide with a subscript indicating the SNP position (HC-Pro: 1-7; CP: 8-15). Bootstrap values indicated at applicable nodes.

III - Principle component analysis

Little clustering is found in the PCA analysis of both the 2008 and 2009 field trials (Figures 5 and 6, respectively). The Sidney 81, Type, and El Batan 3 strains, at opposite sides of the graph, represent the degree of variation that may be found among WSMV strains. In the 2008 trial, most samples are located between the Type and Sidney 81 strains. The starting inoculum is near the bottom center of the graph. Half of the February samples cluster away from the starting inoculum near Type, suggesting a non-random shift in viral variant composition. However, the May samples fall closer to the starting inoculum, with some extending beyond it near Sidney 81. The lack of treatment clustering, with the exclusion of a weakly formed cluster of the late fungicide group, suggests that the variation generated over the growing season is not correlated

with plot treatment, a conclusion further supported by the widespread distribution of the control samples.

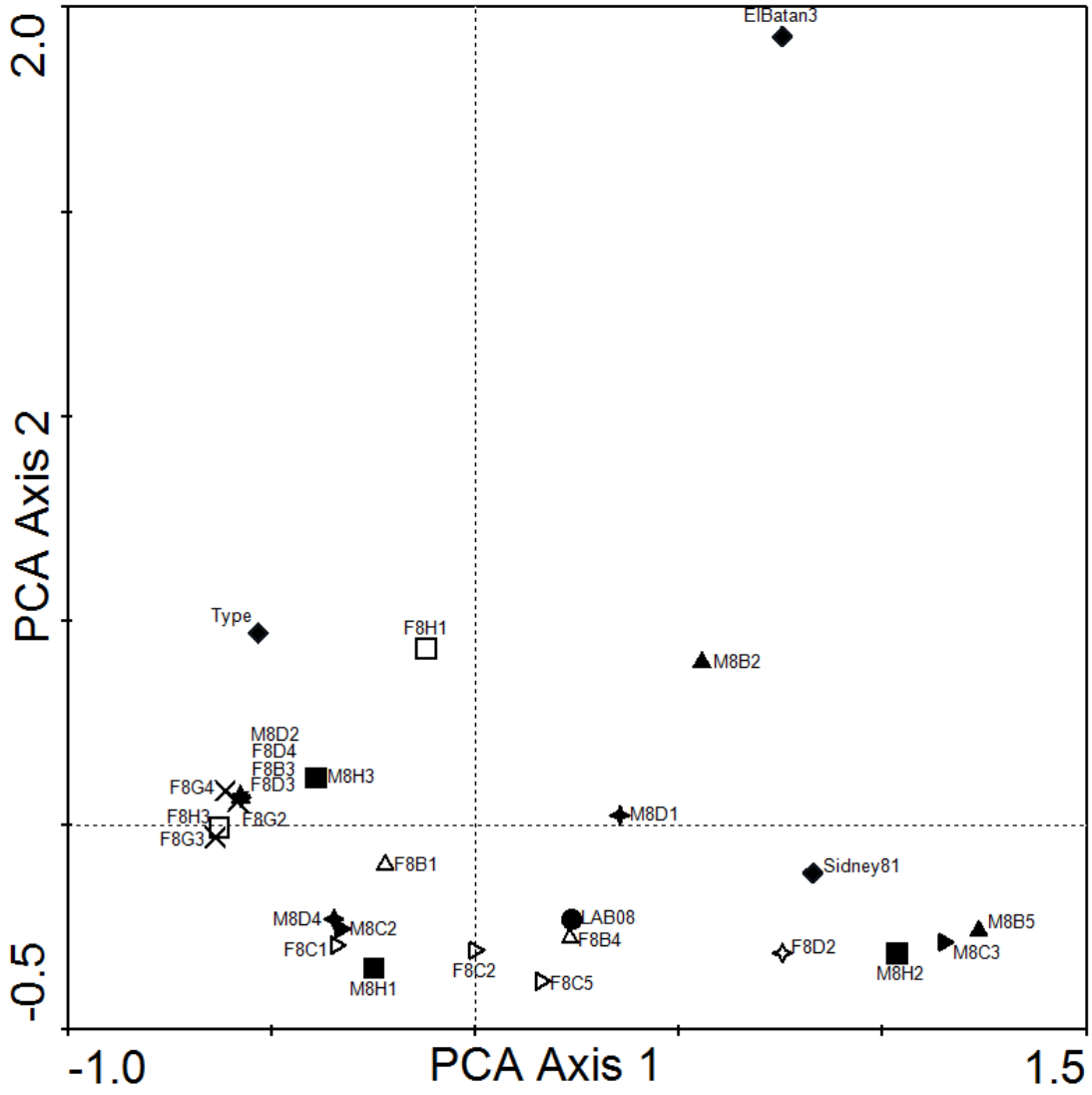

Figure 5: Principle component analysis of WSMV SNP profiles from 2008 field study. The starting inoculum (LAB08) is denoted by a filled circle and the characterized strains of WSMV denoted by filled diamonds. The treatments are denoted by symbols (up-triangle=E&L fungicide, right-triangle=late fungicide, star=control, x-mark=fertilizer, square=insecticide) and the months denoted by a filled (May) and unfilled (February) symbol.

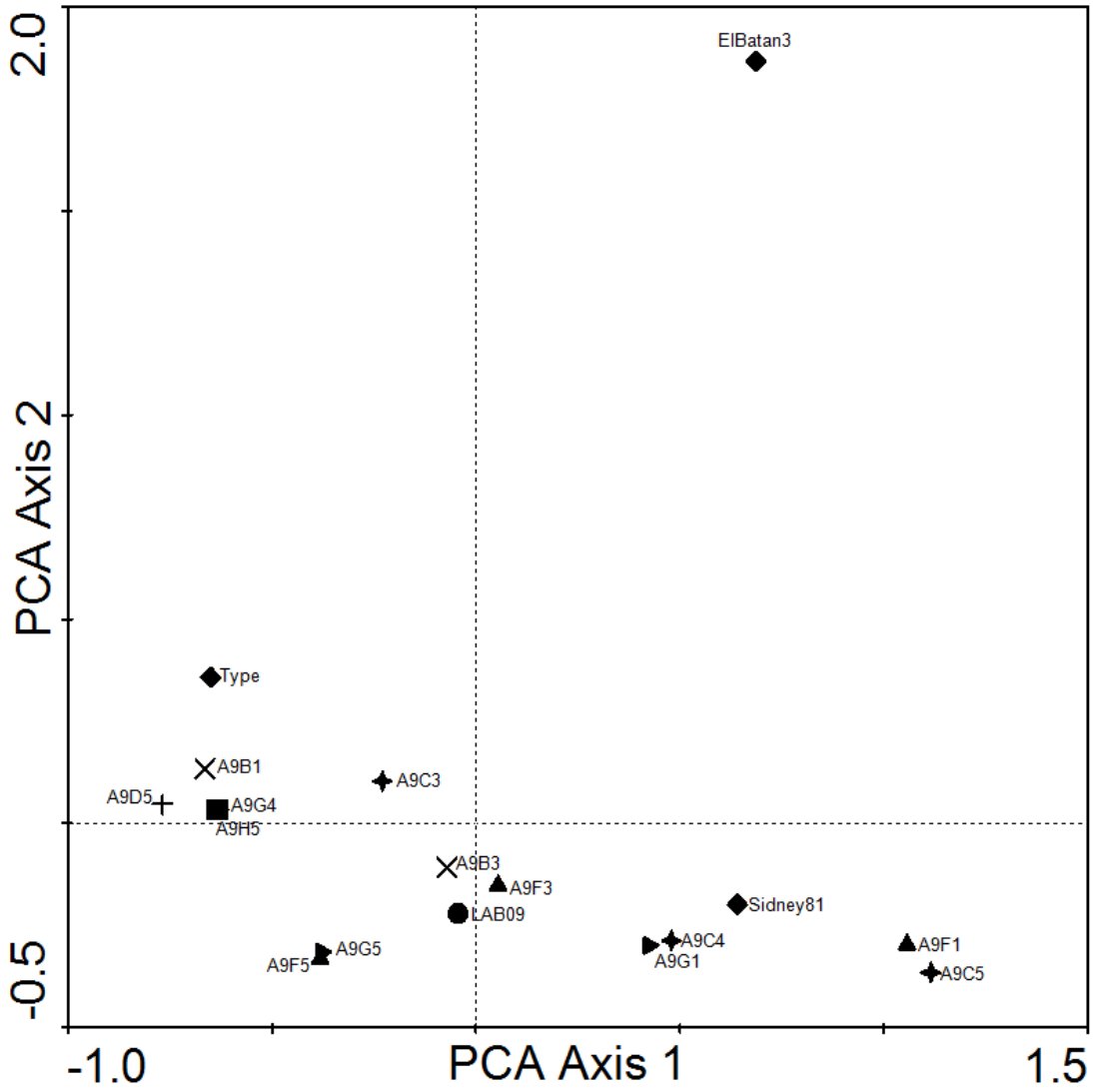

Figure 6: Principle component analysis of WSMV SNP profiles from 2009 field study. The starting inoculum (LAB09) is denoted by a filled circle and the characterized strains of WSMV denoted by filled diamonds. The treatments are denoted by symbols (up-triangle=E&L fungicide, right-triangle=late fungicide, star=control, x-mark=fertilizer, square=insecticide, cross=shade).

The 2009 field trial PCA depicts relationships similar to those of the 2008 trial. Without the inclusion of an earlier month's data, comparable to the February samples in the 2008 trial, I cannot confirm the change of the viral relationships from an early cluster near Type to a spread out distribution near Sidney 81. However, there are several April samples in the same location as the 2008 February cluster, which suggests that a similar phenomenon would have occurred in

2009. The late fungicide group does not form a cluster in 2009 like that observed in 2008, suggesting the difference in heterogeneity is not a response to the fungicide treatment.

IV - Statistical analysis

The amount of each nucleotide present in each SNP position (expressed as percent of total fluorescence) was compared to the nucleotide percentages of the other samples within the same treatment group using ANOVA. After analysis, the values for each nucleotide were compiled to generate an overall analysis of variance, as seen in Table 7. All four nucleotides had a Pr value >0.05, indicating that there is no statistically significant difference among the treatments and starting inoculum. Figure 7 also shows the lack of correlation among the mean estimates of each nucleotide to field treatment. These results further suggest that the SNP profile is not affected by the treatments applied to the wheat field.

Table 2: ANOVA results for 2008 and 2009 field samples at α=0.05

Effect	Nucleotide	Num DF*	Den DF+	F Value	Pr > F±
Treatment	A	7	248	1.10	0.3621
Treatment	C	7	271	0.76	0.6232
Treatment	G	7	245	0.93	0.4849
Treatment	T	7	280	1.37	0.2193

*Num DF= Numerator degrees of freedom

+Den DF= denominator degrees of freedom

±Pr greater than 0.05 indicates statistically significant differences

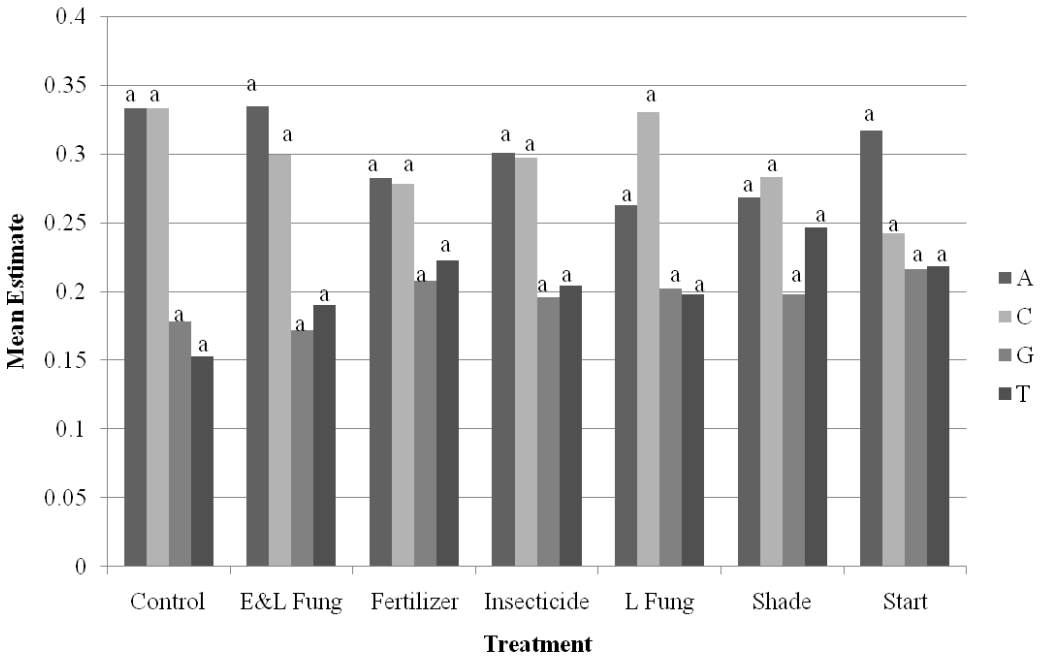

Figure 7: Mean estimate of 2008 and 2009 field samples from ANOVA analysis, based on the percent of total fluorescence for each nucleotide within each SNP position. Letters above the bars indicates the level of significance.

Discussion

The SNP profiles generated for each field sample demonstrate divergence of the viral population over the growing season. While considerable variation was detected, the majority came from changes in minor variants of the population as illustrated by the consistency of the major viral variant profile in Figure 2. This demonstrates that the pathogen variation did not hamper the interpretation of the SNP profiling. This finding is consistent with those of previous studies demonstrating that mutants are produced but may not become established due to genetic and physical bottlenecks (Choi et al, 2001; French and Stenger, 2003; Hall et al, 2001a, 2001b).

The data from this study, including the IUB sequence comparison, the phylogenetic analysis, the principle component analysis and the lack of statistically significant variation differences among the treatment plots, all suggest that the field treatments I applied did not significantly affect the WSMV SNP profile. Therefore, forensic comparisons of WSMV from a

field sample should remain unaltered from agronomic treatments that may have been applied by the producer during the growing season. Understanding this relationship, or lack thereof, between field treatments and viral genotype will help to develop a sampling strategy for forensic evidence by reducing concerns of environmental influences. Similar levels of variation were found in the control plots as were in the treated plots, indicating that the observed variations are due to the natural variation of the virus and not to selection pressures generated by the treatments. However, this study was conducted in only one location over a two-year period using one variety of wheat. Expanding the study to include multiple varieties of wheat, increasing the sample size and study duration, and incorporating fields in different climates would provide a more thorough understanding of the genetic variations that may occur due to environmental or physiological changes.

Interestingly, no WSMV positives were found in the May 2008 fertilizer plot although it was present in February. A conclusion that the fertilization decreased the wheat's susceptibility to the viral infection was not supported by the April 2009 fertilizer treatment results, in which two of five samples were positive. Additionally, a large portion of the February samples in the 2008 field trial clustered away from the starting inoculum in the PCA analysis (Figure 5), suggesting there was a non-random change to a specific genotype. However, the genetic changes that occurred from February to May resulted in the profiles mutating back towards the starting inoculum with a larger degree of variation than that observed in February. It is not surprising to find more variation in May than February because more time was available for mutants to arise and replicate. However, having half of the samples change to a specific genotype dissimilar to the starting inoculum was unexpected, and the reason for it remains unclear.

The widespread distribution of the field trial samples in the PCA analysis is consistent with previous studies in which WSMV genotypes in the Great Plains region of the U.S. were found to be comprised of a single population (Choi et al, 2001; McNeil et al, 1996). French and

Stenger (2005) demonstrated that virus populations derived from a single founding event can vary due to the bottlenecks imposed during transmission and movement, and from the genetic isolation created from cross-protection. This explanation is probably relevant also to the variation noted in this study, with the exception of the transmission bottleneck component, because the WSMV vector, *Aceria tulipae* (Keifer), has not been found near the location of the field trials. If the vector had been present in the field, wheat outside of the inoculated area would most likely have been infected, but samples from the buffer control subplots were negative.

In conclusion, the agronomic and environmental treatments applied to the wheat field in this study did not significantly affect the genetic profile of WSMV, as determined by the application of a SNaPshot assay for WSMV isolate discrimination. Even though the major viral variant in the population was maintained throughout the growing season, the genetic profiles varied among the samples. Such variability needs to be considered when sampling an infected field for assessment of pathogen genetic composition. Further studies are needed, with WSMV and other plant pathogens, to develop strategic sampling plans that accurately represent the pathogen genetic variability in the field.

Acknowledgments

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LITERATURE CITED

Blandino, M., and Reyneri, A. 2009. Effect of fungicide and foliar fertilizer application to winter wheat at anthesis on flag leaf senescence, grain yield, flour bread-making quality and DON contamination. Eur. J. Agron. 30:275-282.

Bockus, W. W. 2010. Compendium of wheat diseases and pests (3rd ed., pp. viii, 171 p.). St. Paul, Minn.: APS Press.

Budowle, B., Burans, J. P., Breeze, R., Wilson, M., and Chakraborty, R. 2005. Microbial Forensics. In R. Breeze, B. Budowle and S. E. Schutzer (Eds.), Microbial Forensics (pp. 1-26). San Diego, CA: Elsevier Academic Press.

Budowle, B., Johnson, M. D., Fraser, C. M., Leighton, T. J., Murch, R. S., and Chakraborty, R. 2005. Genetic analysis and attribution of microbial forensics evidence. Crit. Rev. Microb. 31:233-254.

Budowle, B., Murch, R., and Chakraborty, R. 2005. Microbial forensics: the next forensic challenge. Int. J. Legal Med. 119: 317-330.

Budowle B., Schutzer S.E., Ascher M.S., Atlas R.M., Burans J.P., Chakraborty R., Dunn J.J., Fraser C.M., Franz D.R., Leighton T.J., Morse S.A., Murch R., Ravel J., Rock D.L., Slezak T.R., Velsko S.P., Walsh A.C., and Walters R.A. 2005. Toward a system of Microbial Forensics: from sample collections to interpretation of evidence. Appl. Environ. Microbiol. 71:2209-2213.

Budowle B., Schutzer S.E., Burans J.P., Beecher D.J., Cebula T.A., Chakraborty R., Cobb W.T., Fletcher J., Hale M.L., Harris R.B., Heitkamp M.A., Keller F.P., Kuske C., LeClerc J.E., Marrone B.L., McKenna T.S., Morse S.A., Rodriquez L.L., Valentine N.B., and Yadev J.

2006. Quality sample collection, handling, and preservation for an effective microbial forensics program. Appl. Environ. Microbiol. 72:6431-6438.

Budowle B., Schutzer S.E., Einseln A., kelley L., Walsh A.C., Smith J., Marrone B.L., Robertson J., and Campos J. 2003. Public health: Building microbial forensics as a response to bioterrorism. Science 301:1852-1853.

Choi, I.-R., Hall, J. S., Henry, M., Zhang, L., Hein, G. L., French, R., and Stenger, D. C. 2001. Contributions of genetic drift and negative selection on the evolution of three strains of *Wheat streak mosaic tritimovirus*. Arch. Virol. 146:619-628.

Crooks, G., Hon, G., Chandonia, J., and Brenner, S. 2004. WebLogo: a sequence logo generator. Genome Res. 14:1188-1190.

Cummings, C. A., and Relman, D. A. 2002. Microbial forensics - "cross-examining pathogens". Science 296:1976-1979.

Cupp, O. S., Walker II, D. E., and Hillison, J. 2004. Agroterrorism in the US: Key security challenge for the 21st century. Biosecur. Bioterror. 2:97-105.

Fletcher J., Bender C., Budowle B., Cobb W.T., Gold S., Ishimaru C., Luster D., Melcher U., Murch R., Scherm H., Seem R., Sherwood J.L., Sobral B., and Tolin S. 2006. Plant pathogen forensics: capabilities, needs, and recommendations. Microbiol. Mol. Biol. Rev. 70:450-471.

French, R., and Stenger, D. C. 2003. Evolution of *Wheat streak mosaic virus*: Dynamics of population growth within plants may explain limited variation. Annu. Rev. Phytopathol. 41:199-214.

French, R., and Stenger, D. C. 2005. Population structure within lineages of *Wheat streak mosaic virus* derived from a common founding event exhibits stachastic variation inconsistent with the deterministic quasi-species model. Virology 343:179-189.

Grossmann, K., and Retzlaff, G. 1997. Bioregulatory Effects of the Fungicidal Strobilurin Kresoxim-methyl in Wheat *(Triticum aestivum)*. Pesticide Sci. 50:11-20.

Hall, J. S., French, R., Hein, G. L., Morris, T. J., and Stenger, D. C. 2001. Three distinct mechanisms facilitate genetic isolation of sympatric *Wheat streak mosaic virus* lineages. Virology 282: 230-236.

Hall, J. S., French, R., Morris, T. J., and Stenger, D. C. 2001. Structure and temporal dynamics of populations within *Wheat streak mosaic virus* isolates. J. Virol. 75:10231-10243.

Hunger, R. M., Sherwood, J. L., Evans, C. K., and Montana, J. R. 1992. Effects of planting date and inoculation date on severity of wheat streak mosaic in hard red winter wheat cultivars. Plant Dis. 76:1056-1060.

Krieg, U., Weile, M., and Gohlich, F. 2003. Experience of cereal disease control with Stratego and Twist in Germany. Pflanzenschutz-Nachrichten Bayer 56:297-312.

McKinney, H. H. 1937. Mosaic diseases of wheat and related cereals. US Department of Agric.Circ. 442:1-23.

McNeil, J. E., French, R., Hein, G. L., Baenziger, P. S., and Eskridge, K. M. 1996. Characterization of genetic variability among natural populations of *Wheat streak mosaic virus*. Phytopathology 86:1222-1227.

Molyneux, D. E., and McKinlay, R. G. 1989. Observations on germination and acetylcholinesterase activity in wheat seeds. Annals of Botany 63:81-86.

Nomenclature Committee of the International Union of Biochemistry (NC-IUB). 1986. Nomenclature for incompletely specified bases in nucleic acid sequences: recommendations 1984. . Proceedings of the National Academy of Sciences, 83:4-8.

Stenger, D. C., Hall, J. S., Choi, I.-R., and French, R. 1998. Phylogenetic relationships within the family *Potyviridae*: *Wheat streak mosaic virus* and *Brome streak mosaic virus* are not members of the genus *Rymovirus*. Phytopathology 88:782-787.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. *submitted*.

ter Braak, C., and Smilauer, P. 2002. Canoco Reference Manual and CanoDraw for Windows User's Guide: Software for Canonical Community Ordination (version 4.5). Ithaca, NY: Microcomputer Power.

US Wheat Associates. (2010) U.S. wheat associations annual report 2009/10.

Walker, A. S., Auclair, C., Gredt, M., and Leroux, P. 2009. First occurrence of resistance to strobilurin fungicides in Microdochium nivale and Microdochium majus from French naturally infected wheat grains. Pest Manag. Sci. 65:906-915.

CHAPTER VI

EVALUATING *WHEAT STREAK MOSAIC VIRUS* ISOLATE RELATIONSHIPS USING SINGLE NUCLEOTIDE POLYMORPHISMS

Abstract

Determining the spatial spread of plant pathogens is an important aspect to epidemiological research and can also be vital to a forensic investigation of a plant disease event. Satellite imagery can be used for identifying outbreak epicenters and tracing the spatial spread, but molecular typing can be more discriminatory by identifying the genetic changes occurring in a virus population. Current virus typing methods include sequencing, amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP), multi-locus variable number tandem repeat analysis (MLVA) and single nucleotide polymorphisms (SNPs), all of which provide different levels of discriminatory power. Using SNP typing, the spatial spread of WSMV field isolates from two distinct geographical regions in the U.S. was assessed. Principle component and phylogenetic analyses showed that each isolate produced a unique SNP profile, but I found no correlation between genotype and location, most likely because WSMV is a well-established virus nearing genetic saturation in the United States. However, information from the SNP profiles may be relevant to forensic investigation and could lead to attribution of a crime.

Introduction

Plant pathogens are a serious risk to crop production throughout the world, causing an average annual yield reduction of 14% (Agrios, 2005; Oerke, 2006; Strange and Scott, 2005). Monitoring the spatial spread of pathogens, especially those that are exotic or newly introduced, may facilitate disease prevention or response, and also may be applicable to the forensic investigation of plant pathogen use in a biocriminal or bioterrorist act, when source identification can inform criminal attribution (Budowle et al, 2005; Fletcher et al, 2006).

Common tools for collecting spatial disease data for epidemiological studies of plant pathogens include geographic information systems, global positioning systems, and remote sensing (Maffei and Arena 1992; Nilsson, 1995; Pozdnyakova et al, 2000). While satellite imagery and mapping also can be useful for tracing the spread of a pathogen, detecting genetic changes can enhance the discriminatory power for attribution of a crime (Fletcher et al, 2006). Some common molecular typing techniques include sequencing, restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), multiple locus variable number tandem repeat analysis (MLVA), and single nucleotide polymorphisms (SNPs) (Budowle et al, 2005; Fletcher et al, 2006; Vincelli and Tisserat, 2008). Of these, nucleotide sequencing provides the greatest amount of genetic information. However, while the cost of sequencing is becoming more reasonable, data analysis is extensive, requiring trained individuals and considerable time. RFLP and AFLP are commonly used for DNA fingerprinting and population genetics studies (Bayles et al, 2001; Hong et al, 1996; Newton et al, 2010; Picado et al, 2011; Restrepo et al, 1999; Saleh et al, 2010; Singru et al, 2003). However, they are less effective if the sample DNA or RNA is severely degraded and cannot identify specific genetic changes. MLVA is a powerful tool that utilizes repeated regions of microbial genomes for strain identification, as demonstrated following the 2001 anthrax attack (Keim et al, 2000, 2004). For organisms lacking repeat regions, such as viruses, or for comparing isolates of low genetic

diversity, SNP typing is more appropriate than other methods and can be employed even when the nucleic acid is degraded (Budowle et al, 2005; Keim et al, 2004).

Wheat streak mosaic virus (WSMV), a common pathogen of wheat in the United States and many other wheat growing regions of the world, is a single stranded, positive sense RNA virus belonging to the family *Potyviridae* and genus *Tritimovirus* (Stenger et al, 1998, 2002). The virus is transmitted by *Aceria tulipae* Keifer, the wheat curl mite (Connin, 1956; Murray et al, 2005; Somsen and Sill, 1970; Stenger et al, 2005). The genetic diversity of WSMV populations in the U.S. has been well studied since the first documented disease occurrence in the early 1920s (McKinney, 1937). The Protein 1 (P1), Protein 3 (P3), and the coat protein (CP) genes are the most variable (Chenault et al, 1996; Choi et al, 2001, 2002, 2005; French and Stenger, 2003, 2005; Gadiou et al, 2009; Hall et al, 2001a, 2001b; McNeil et al, 1996; Niblett et al, 1991; Stenger et al, 1998, 2002), and the variability within the CP gene is representative of the overall genomic variability (Shukla, Frenkel, and Ward, 1991). Studies comparing WSMV populations within the U.S. showed the same levels of viral divergence among plants within a single field as among plants in a state, suggesting that WSMV in the U.S. is actually a single population (French and Stenger, 2003, 2005; Rabenstein, Seifers, Schubert, French, and Stenger, 2002).

A SNP typing method was recently developed for discriminating among strains of WSMV (Chapter IV) (Carver, 2007). In this study, I evaluated the usefulness of WSMV SNP typing for evaluating the spatial spread of the virus. Using geographically distinct WSMV outbreaks, genetic fingerprints of field isolates were generated and compared using principle component analysis (PCA) and phylogenetic analyses. The accuracy of the genetic variability determined by the SNP typing method was evaluated by comparing the results to the genetic relatedness determined by CP gene sequencing.

Materials and Methods

I - Selection of virus samples

WSMV isolates, obtained from the Great Plains Diagnostic Network (GPDN), associated with the National Plant Diagnostic Network (Stack et al, 2006, 2007; Stack and Fletcher, 2007), wheat virus survey of 2007 and 2008, were selected from Montana and the panhandle regions of Texas and Oklahoma (Table 1). Nine Montana samples were collected from separate fields near six cities across that state. Eighty Oklahoma and Texas panhandle samples were collected from separate fields in eleven counties across the two states. The presence of WSMV had been confirmed by the GPDN laboratories in the respective states using PCR and ELISA. All samples were stored as leaf tissue at -80°C until sample processing.

Wheat tissue infected with WSMV strains Sidney 81 and Type were received from Roy French (University of Nebraska-Lincoln) and stored at -80°C. I was unable to obtain tissue infected with the El Batan 3 strain, thus the sequence of this strain was downloaded from GenBank (Accession: AF285170.1) and the *in silico* results were used for the analyses. *Oat necrotic mottle virus* (ONMV) was chosen as the outgroup for the phylogenetic analysis and the SNP profile generated *in silico* from its sequence in GenBank (Accession: NC_005136.1).

II - Nucleic acid extraction

Total RNA was extracted from 100 mg of frozen leaf tissue using the RNeasy Plant Mini Extraction Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. The tissue was homogenized using a mini bead-beater (Biospec Products, Bartlesville, OK) and 550 µl of RLT lysis buffer. The RNA was eluted using 50 µl of RNase-free water and the concentrations were determined using a Nanodrop Spectrophotometer 2000 (Thermo Scientific, Waltham, MA).

Table 3: WSMV samples from GPDN wheat virus survey

Sample ID	State	County	Year collected
Billings	MT	Yellowstone	2007
Conrad 1	MT	Pondera	2007
Conrad 2	MT	Pondera	2007
Conrad 3	MT	Pondera	2007
Helena	MT	Lewis and Clark	2007
Huntley A	MT	Yellowstone	2007
Huntley B	MT	Pondera	2007
Kalispell	MT	Flathead	2007
Toole	MT	Mineral	2007
HAN1	TX	Hansford	2008
HAN2	TX	Hansford	2008
HAN3	TX	Hansford	2008
HAN4	TX	Hansford	2008
OCH1	TX	Ochiltree	2008
OCH2	TX	Ochiltree	2008
POT1	TX	Potter	2008
POT2	TX	Potter	2008
POT3	TX	Potter	2008
POT4	TX	Potter	2008
POT5	TX	Potter	2008
OLD1	TX	Oldham	2008
OLD2	TX	Oldham	2008
DS1	TX	Deaf Smith	2008
DS2	TX	Deaf Smith	2008
DS3	TX	Deaf Smith	2008
DS4	TX	Deaf Smith	2008
DS5	TX	Deaf Smith	2008
RAN1	TX	Randall	2008
RAN2	TX	Randall	2008
RAN3	TX	Randall	2008
RAN4	TX	Randall	2008
RAN5	TX	Randall	2008
RAN6	TX	Randall	2008
RAN7	TX	Randall	2008
RAN8	TX	Randall	2008
RAN9	TX	Randall	2008
RAN10	TX	Randall	2008
RAN11	TX	Randall	2008
RAN12	TX	Randall	2008
RAN13	TX	Randall	2008
RAN14	TX	Randall	2008
RAN15	TX	Randall	2008
RAN16	TX	Randall	2008
PAR1	TX	Parmer	2008
PAR2	TX	Parmer	2008
CAS1	TX	Castro	2008
CAS2	TX	Castro	2008
CAS3	TX	Castro	2008
CAS4	TX	Castro	2008
HAL1	TX	Hale	2008
HAL2	TX	Hale	2008
HAL3	TX	Hale	2008
HAL4	TX	Hale	2008
HAL5	TX	Hale	2008
TEX1	OK	Texas	2008
TEX2	OK	Texas	2008
TEX3	OK	Texas	2008
TEX4	OK	Texas	2008
TEX5	OK	Texas	2008
BEA1	OK	Beaver	2008
BEA2	OK	Beaver	2008
BEA3	OK	Beaver	2008
BEA4	OK	Beaver	2008
BEA5	OK	Beaver	2008

III - RT-PCR

The complementary DNA (cDNA) of the CP and helper component-protease (HC-Pro) genes and flanking regions of each virus strain were synthesized and amplified in separate reactions using a SuperScript™ III One-Step RT-PCR kit with Platinum© *Taq* (Invitrogen, Carlsbad, CA). The reaction and post-extension treatment were carried out as described in Chapter IV on page 79.

The amplification of the CP and HCPro genomic regions was confirmed by electrophoresis using a 1.5% agarose gel containing SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, CA) and 1X TAE buffer. The samples and a 100 bp DNA ladder (Invitrogen, Carlsbad, CA) were loaded in the gel using 1X loading dye containing bromophenol blue and xylene cyanol and were electrophoresed for 45 minutes at 100mV/cm. The gel was visualized and photographed using ultraviolet radiation.

IV - Single base extension PCR

Genetic fingerprints of the viral isolates were generated using the PRISM® SNaPshot™ Multiplex Kit (Applied Biosystems, Inc., Foster City, CA). Fluorescently labeled terminator nucleotides were incorporated at known single nucleotide polymorphism locations during PCR, following the procedure described in Chapter IV on page 80.

V - Sequencing

Virus cDNA from each of the Montana samples was sequenced three times in both directions using a Big Dye Terminator Kit v1.0 (Applied Biosystems, Inc., Foster City, CA) and the C1 and XC1 primers (Carver, 2007). Sequence corrections were made manually using Sequence Scanner v1.0 (Applied Biosystems, Inc., Foster City, CA). Alignments of the forward and reverse sequences were completed using EMMA (Rice et al, 2000), and the consensus

sequence was generated using CONS (Rice et al, 2000) with manual inspections of accuracy. All consensus sequences were aligned using ClustalW (EMBL-EBI, Cambridge, UK) and trimmed manually to the same length (990 nt).

VI - Data analysis

The area under each fluorescent peak higher than 800 FU was determined and the nucleotide percentages calculated for each sample at each SNP position using Microsoft Excel (Microsoft, Redman, WA). The cutoff of 800 FU was previously established during the development of the WSMV SNaPshot method, described in Chapter IV. A principle component analysis (PCA) with square-root transformation was performed using Canoco software (Biometris, Wageningen UR, The Netherlands) to assess the variability among the samples.

An unweighted pair group method with arithmetic mean (UPGMA) tree was created using the Jukes-Cantor model for the Montana consensus sequences. Analysis using MEGA 5.0 (Tamura et al, 2011) included the comparable ONMV, Sidney 81, Type, and El Batan 3 sequences.

Results

I - Amplification of WSMV

The CP and HCPro genes were amplified from all nine MT samples and 56 of the 80 OK and TX panhandle samples (Figure 1). Presence of the WSMV characteristic ~1.3 kb amplicon in the positive control for CP and HCPro and the absence of amplicons in the negative control demonstrated that the amplicons were from WSMV in the sample and not from contamination.

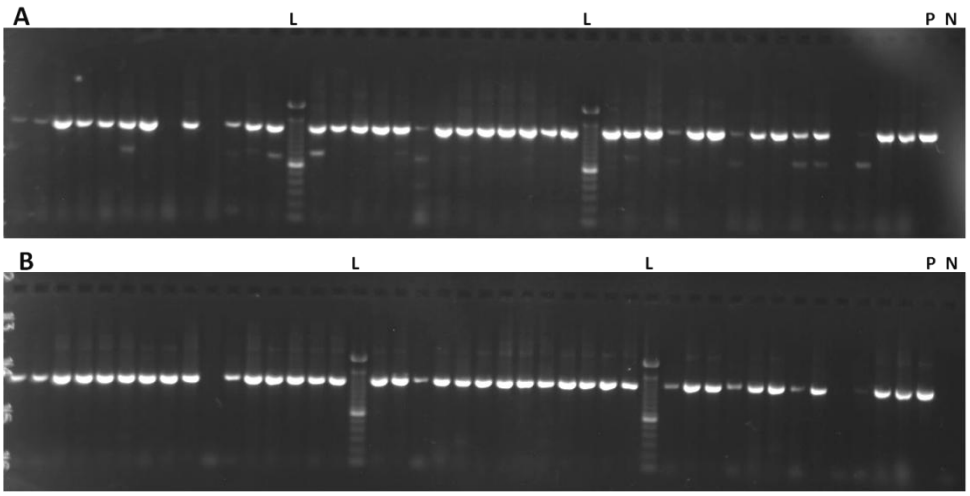

Figure 1: Gel image of select WSMV field isolates showing characteristic ~1.3 kb amplicon for both the CP (A) and HC-Pro (B) genes. Wells labeled L, P, and N are the 100bp DNA Ladder, positive control plasmid, and negative control, respectively.

II - Genetic accuracy of SNP profile

To apply the SNaPshot method to assess the variability among WSMV field isolates, its ability to produce a reliable and accurate representation of isolate genotypes was evaluated. The variability of generated SNP profiles for the Montana samples was compared using PCA (Figure 2). The CP amplicon for these samples was sequenced and a phylogenetic analysis was performed using the consensus sequences (Figure 3). The relationship depicted in the UPGMA tree of the consensus sequences is consistent with the relationship depicted in the PCA, suggesting the SNaPshot method generates an accurate representation of the isolate genotype. The nucleotides present in the SNP profile were all present in the consensus sequences, even the minor peaks, further demonstrating the SNP profile is accurately identifying the genotype of the virus (data not shown).

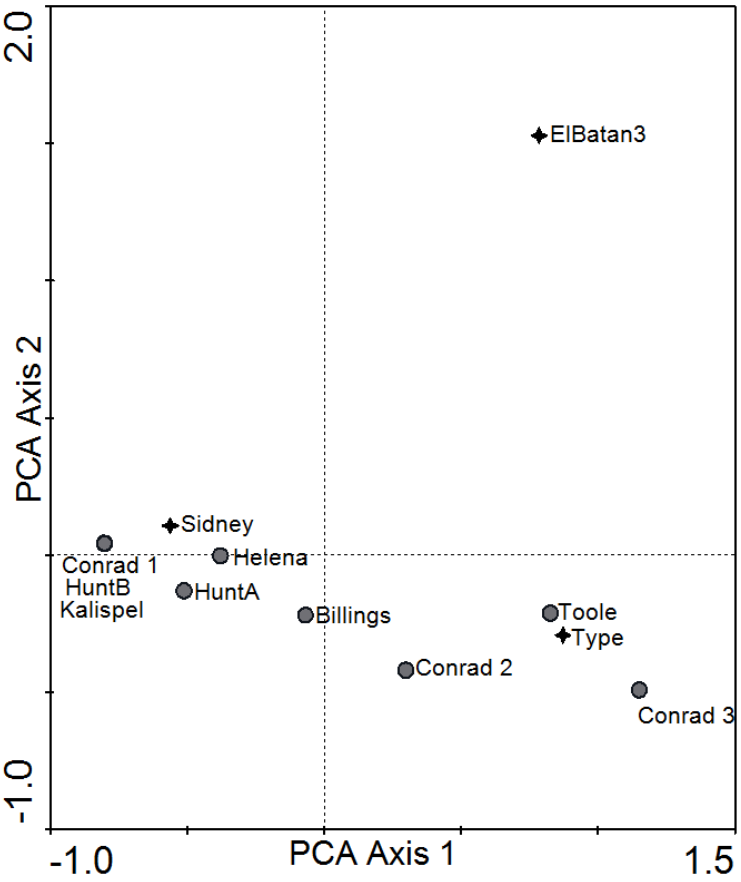

Figure 2: Principle component analysis of Montana WSMV isolates. Each sample is represented with a solid circular symbol. The Sidney 81, Type, and El Batan 3 isolates were included as reference samples and are denoted with a star symbol.

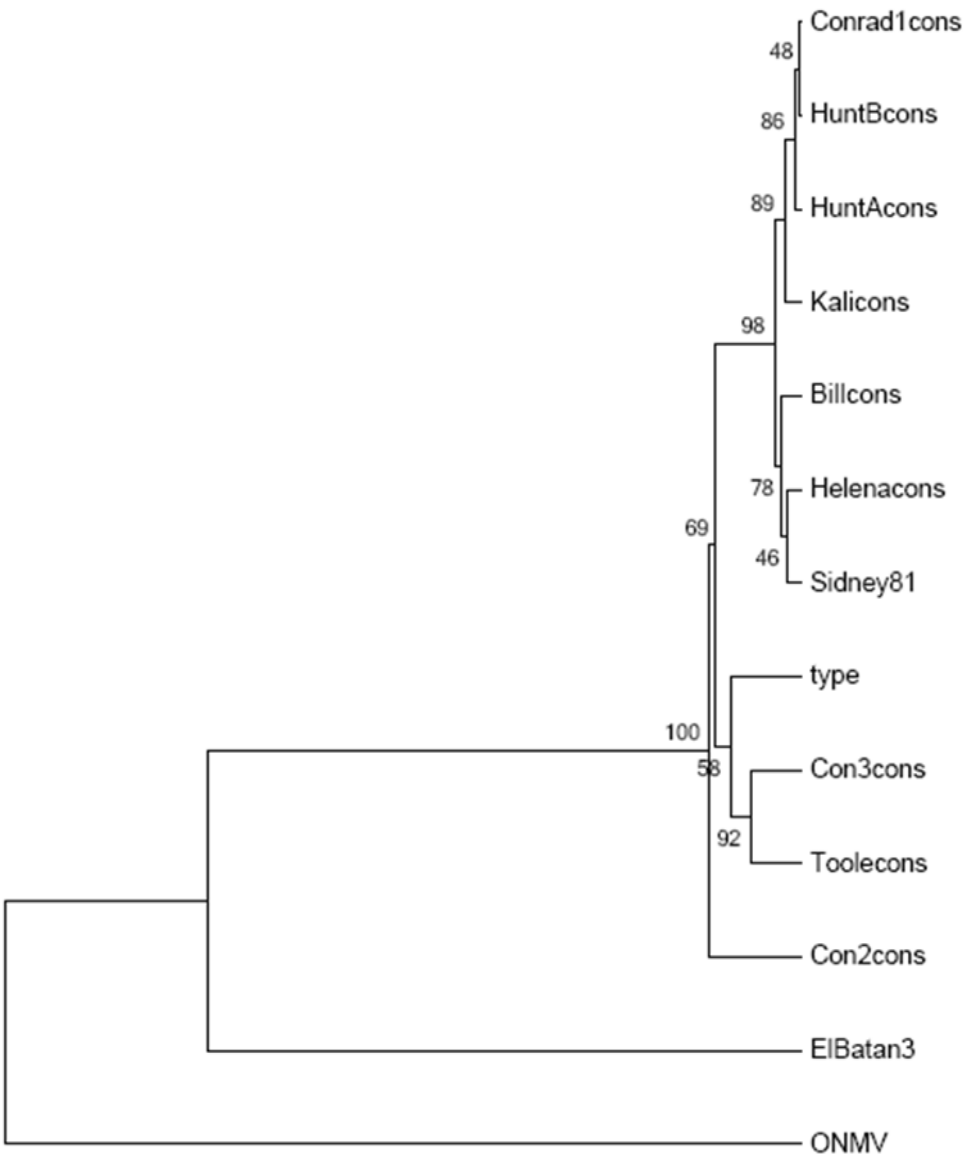

Figure 3: UPGMA tree of the Montana WSMV isolate consensus sequences using the Jukes-Cantor model, generated using MEGA 5.0. The tree is rooted at ONMV and 1000 bootstrap replicates were performed.

III - Isolate relationships

The PCA results for the Montana WSMV isolates (Figure 2) shows a wide genomic distribution for most of the samples, with the exception of a small cluster containing Conrad 1, Kalispell, and Huntley B. Conrad 1, Conrad 2, and Conrad 3 are scattered on the graph, even though they were all collected near the city of Conrad, MT. A comparison of the PCA results

with a topographic map of Montana shows no correlation between viral genotype and geographic location. A WSMV isolate from the city of Toole, MT, which is located on the far west side of the state and isolated by the Rocky Mountains extending north and south, appears to be the most divergent from the other viral isolates. However, the genotype of a viral isolate from Kalispell, MT, also located within the mountain range, is very similar to Conrad 1 (100 miles east) and Helena (200 miles southeast), both of which lie outside of the mountain range. The cities of Billings and Huntley are the closest geographically (15 miles apart), and the genotypes of viruses collected in these two locations were similar (although not the most similar among all the comparisons).

The PCA results of WSMV isolates from OK and the TX Panhandle (Figure 4) show results similar to those from Montana, with no correlation evident between geography and genotype. The eleven counties represented by the samples are located in the Texas panhandle and of the Oklahoma panhandle area due north of the Texas counties, encompassing approximately $5.2x10^7$ km^2. Most of these viruses were spread out across the PCA graph, but those from four Texas counties (Ochiltree, Hansford, Parmer, and Hale) were found only in the densest area of the graph. Ochiltree and Hansford Counties are on the northern edge of the Texas panhandle, just south of Beaver and Texas Counties in Oklahoma (Figure 5). Parmer and Hale Counties are in the mid-west region of the Texas panhandle, and are the southern-most counties included in this study. The remaining samples collected from countries located between these two extremes and the samples from Oklahoma all have representatives in the densest area of the graph, but also have at least one outlier.

When the Montana, Oklahoma, and Texas samples were included in a single PCA analysis (Figure 6), there was no overlap between the Montana isolates and the Oklahoma/Texas isolates. However, the Montana samples are more similar to the Oklahoma/Texas samples and to

the Type and Sidney 81 strains than to El Batan 3, the most divergent strain of WSMV (Rabenstein et al, 2002).

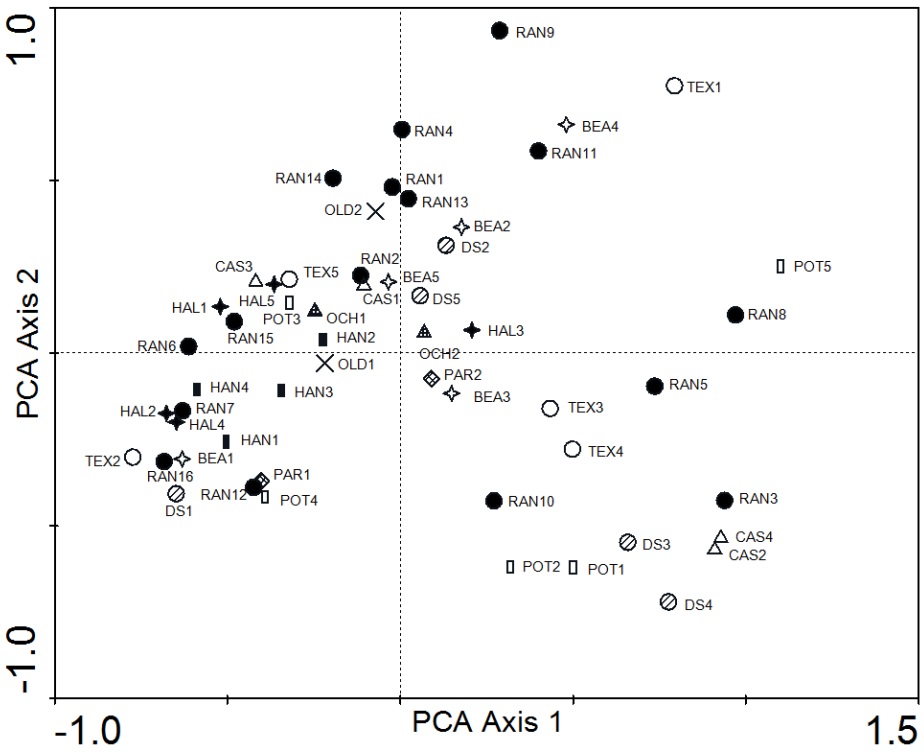

Figure 4: Principle component analysis of TX and OK Panhandle WSMV isolates. Each county is represented by a unique symbol, the number depending on the sample number from that county. County codes: HAN=Hansford Co, OCH=Ochiltree Co, POT=Potter Co, OLD=Oldham Co, DF=Deaf Smith Co, RAN=Randall Co, PAR=Parmer Co, CAS=Castro Co, HAL=Hale Co, TEX=Texas Co, BEA=Beaver Co.

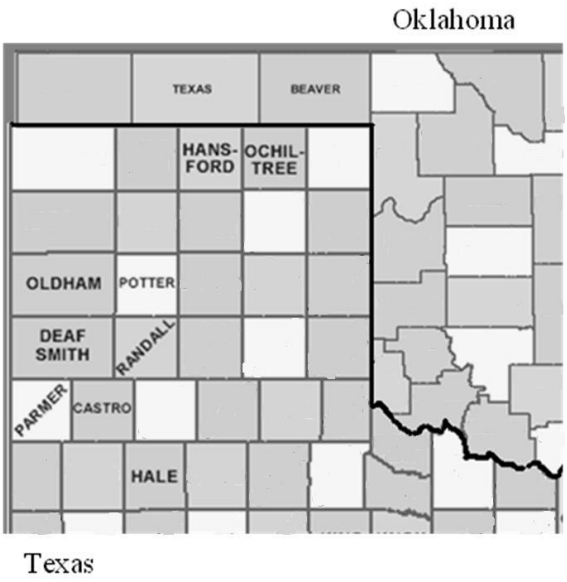

Figure 5: County map of the Texas and Oklahoma Panhandles.

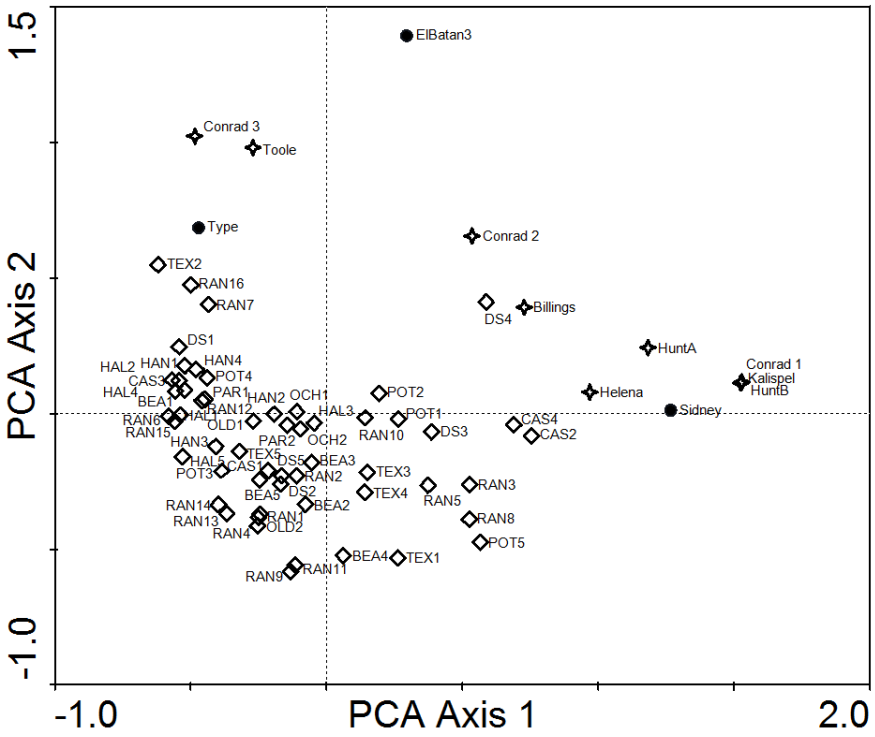

Figure 6: Principle component analysis of Montana, Oklahoma, and Texas field isolates. The stars and diamonds represent the Montana isolates and the Oklahoma/Texas isolates, respectively. The El Batan 3, Type, and Sidney 81 WSMV strains were included to provide a known measure of divergence and are marked with solid circles.

Discussion

If a viral pathogen were to be used in a biocrime, tracing its previous movement could provide clues important to a forensic investigation. Assessing the spatial spread of a plant virus is a complex task involving time consuming and data extensive processes. The SNP profiling method recently developed for WSMV is a simple and quick method for genotype comparisons (Chapter IV).

The two WSMV populations studied here both consisted of highly variable isolates, based on their CP and HCPro gene sequences. Six of the nine Montana samples possessed unique genetic fingerprints by SNP profiling. However, three nearly identical samples were collected from different locations in the state. The Texas/Oklahoma population also showed wide diversity among the viral variants, with similarities among samples from one county being equivalent to those among all panhandle samples. These results are consistent with the theory that U.S. variants of WSMV all belong to a single population (Choi et al, 2001; French and Stenger, 2003). The slight differences found between the Montana and the Oklahoma/Texas panhandle samples could be due to a change in the environment, such as climate, wheat varieties planted, and/or terrain. Alternatively, the finding could be an artifact due to the low sample number of the Montana population. The single population theory can account for the similarities among isolates from these two distinct geographical locations.

However, another explanation may also account for some of the variation. If WSMV were transmitted only by its wind-borne vector, the wheat curl mite, one would expect to see a correlation between virus spread and geography and wind direction. But WSMV can also be transmitted through infected seed, although this occurrence is rare (Hill et al, 1974; Jones et al, 2005; Lanoiselet et al, 2008). With the mass market production of wheat seeds by large corporations and the ever-increasing national and international movement of seeds, the movement

of virus populations to new areas is growing. The recent discovery of WSMV in Australia was attributed to the importation of infected seed from the U.S. Pacific Northwest (Dwyer et al, 2007).

Even though a distinct relationship between location and viral genotype was not identified in this study, the results may still be useful in a forensic investigation. In addition to collecting infected leaf tissue during the investigation, information should be gathered from the producer regarding the crop, including the origin of the seed, the presence of disease in previous years, crops previously planted, importation/exportation of plant material, and presence of insects and other arthropods. Such information can help to identify the origin of the disease, whether it was already present in the field from previous crops or nearby fields or if it was brought in on infected seed or plant material. Finally, the genotypes of viruses in the infected tissue collected at each outbreak can be compared to what is known about the seed origin, field history, and growing season to determine if there is a correlation between the gathered information and the observed genetic changes.

Because WSMV has been well-established in the U.S. for almost 100 years and the population appears to be at genetic saturation (genetic variability within a local area is equivalent to the variability found in a global setting), it is not surprising that a distinctive spatial spread of the virus was not apparent (Choi et al, 2001; Erayman et al, 2003; French and Stenger, 2005). Achon et al (2011) revealed that the *Maize dwarf mosaic virus* (MDMV) genotype could not be correlated with geographical location for in the old maize growing regions of Spain where MDMV has been long established. In contrast, the genetic diversity of MDMV was significantly lower in the recently cultivated maize growing regions of Spain where MDMV has been established only recently, therefore rendering a correlation between genotype and geography. Similar correlations have been drawn for *Turnip mosaic virus* (Ohshima et al, 2002; Tomitaka and Ohshima, 2006). Even though geographical correlations could not be identified in our study,

SNP profiling was an effective way to distinguish among field isolates, making it an appropriate genotyping method (Chapter IV and V). The application of this tool to assess spatial spread of a virus may be more suitable for a virus that was newly introduced or remained in fewer, more isolated geographical locations.

In this study, I evaluated the genetic variability within and between two geographically distinct WSMV populations using a SNP profiling method developed specifically for WSMV. While the genotypes of the two populations were different, the genetic variation observed within each population was high and did not correlate with location. While this method may not precisely distinguish viral populations within a geographical region, it may be useful in forensic investigations.

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LITERATURE CITED

Achon, M. A., Alonso-Dueñas, N., and Serrano, L. 2011. *Maize dwarf mosaic virus* diversity in the Johnsongrass native reservoir and in maize: evidence of geographical, host and temporal differentiation. Plant Pathol.60:369-377.

Agrios, G. E. 2005. Plant Pathology (5th ed.). Oxford, UK: Elsevier Academic Press.

Bayles, R. A., Taylor, E. J. A., Bates, J. A., Hilton, G. J., and Law, J. R. 2001. Virulence variation and molecular polymorphism in *Puccinia striiformis*. Plant Varieties and Seeds 14:143-150.

Budowle, B., Johnson, M. D., Fraser, C. M., Leighton, T. J., Murch, R. S., and Chakraborty, R. 2005. Genetic analysis and attribution of microbial forensics evidence. Crit. Rev. Microbiol. 31:233-254.

Carver, J. 2007. Molecular typing of *Wheat streak mosaic virus* for forensic applications. M.S. Thesis, Oklahoma State Univeristy-Center for Health Sciences, Tulsa, OK.

Chenault, K. D., Hunger, R. M., and Sherwood, J. L. 1996. Comparison of the nucleotide sequences of the coat protein open reading frame of nine isolates of *Wheat streak mosaic rymovirus*. Virus Genes 13:187-188.

Choi, I.-R., Hall, J. S., Henry, M., Zhang, L., Hein, G. L., French, R., and Stenger, D. C. 2001. Contributions of genetic drift and negative selection on the evolution of three strains of *Wheat streak mosaic tritimovirus*. Arch. Virol. 146:619-628.

Choi, I.-R., Horken, K. M., Stenger, D. C., and French, R. 2002. Mapping of the P1 proteinase cleavage site in the polyprotein of *Wheat streak mosaic virus* (genus *Tritimovirus*). J. Gen. Virol. 83:443-450.

Choi, I.-R., Horken, K. M., Stenger, D. C., and French, R. 2005. An internal RNA element in the P3 cistron of *Wheat streak mosaic virus* revealed by synonymous mutations that affect both movement and replication. J. Gen.Virol. 86:2605-2614.

Connin, R. V. 1956. The host range of the wheat curl mite, vector of wheat streak-mosaic. J. Econ. Entomol. 49:1-4.

Dwyer, G. I., Gibbs, M. J., Gibbs, A. J., and Jones, R. A. C. 2007. *Wheat streak mosaic virus* in Australia: relationships to isolates from the Pacific Northwest of the USA and its dispersion via seed transmission. Plant Dis. 91:164-170.

Erayman, M., Baenziger, P. S., French, R., and Hein, G. L. 2003. Application of mobile nursery methods to determine temporal and spatial genetic variability of *Wheat streak mosaic virus* in Nebraska. Cereal Res Commun. 31:105-112.

Fletcher, J., Bender, C., Budowle, B., Cobb, W. T., Gold, S., Ishimaru, C., Luster, D., Melcher, U., Murch, R., Scherm, H., Seem, R.C., Sherwood, J. L., Sobral, B.W., and Tolin, S. 2006. Plant pathogen forensics: capabilities, needs, and recommendations. Microbiol. Mol. Biol. R. 70:450-471.

French, R., and Stenger, D. C. 2003. Evolution of *Wheat streak mosaic virus*: Dynamics of population growth within plants may explain limited variation. Annu. Rev. Phytopathol. 41:199-214.

French, R., and Stenger, D. C. 2005. Population structure within lineages of *Wheat streak mosaic virus* derived from a common founding event exhibits stachastic variation inconsistent with the deterministic quasi-species model. Virology 343:179-189.

Gadiou, S., Kudela, O., Ripl, J., Rabenstein, F., Kundu, J. K., and Glasa, M. 2009. An amino acid deletion in *Wheat streak mosaic virus* capsid protein distinguishes a homogeneous group of European isolates and facilitates their specific detection. Plant Dis. 93:1209-1213.

Hall, J. S., French, R., Hein, G. L., Morris, T. J., and Stenger, D. C. 2001. Three distinct mechanisms facilitate genetic isolation of sympatric *Wheat streak mosaic virus* lineages. Virology 282:230-236.

Hall, J. S., French, R., morris, T. J., and Stenger, D. C. 2001. Structure and temporal dynamics of populations within *Wheat streak mosaic virus* isolates. J. Virol. 75:10231-10243.

Hill, J. H., Martinson, C. A., and Russell, W. A. 1974. Seed transmission of *Maize dwarf mosaic* and *Wheat streak mosaic viruses* in Maize and response of inbred lines. Crop Sci. 14:232-235.

Hong, S. M., Kang, K. Y., Kim, N. S., Kang, S. W., and Kim, H. K. 1996. Random amplified polymorphic DNA and restriction fragment length polymorphism analyses to differentiate races of the rice blast fungus, Pyricularia oryzae in Korea. Mol. Cells 6:346-351.

Jones, R. A. C., Coutts, B. A., Mackie, A. E., and Dwyer, G. I. 2005. Seed Transmission of *Wheat streak mosaic virus* shown unequivocally in wheat. Plant Dis. 89:1048-1050.

Keim, P., Price, L. B., Klevytska, A. M., Smith, K. L., Schupp, J. M., Okinaka, R., Jackson, P. J., and Hugh-Jones, M. E. 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J. Bacteriol. 182:2928-2936.

Keim, P., Van Ert, M. N., Pearson, T., Vogler, A. J., Huynh, L. Y., and Wagner, D. M. 2004. Anthrax molecular epidemiology and forensics: using the appropriate marker for different evolutionary scales. Infect Genet Evol. 4:205-213.

Lanoiselet, V. M., Hind-Lanoiselet, T. L., and Murray, G. M. 2008. Studies on the seed transmission of *Wheat streak mosaic virus*. Australas. Plant Path. 37:584-588.

Maffei, H. M., and Arena, J. V. 1992. Forest insect and disease risk and occurrence maps in GIS: application to integrated resource analysis. Paper presented at the Proceedings: Spatial analysis and forest pest management,Virginia.

McKinney, H. H. 1937. Mosaic diseases of wheat and related cereals. US Department of Agric.Circ. 442: 1-23.

McNeil, J. E., French, R., Hein, G. L., Baenziger, P. S., and Eskridge, K. M. 1996. Characterization of genetic variability among natural populations of *Wheat streak mosaic virus*. Phytopathology 86:1222-1227.

Murray, G. M., Knihinicki, D. K., Wratten, K., and Edwards, J. 2005. Wheat streak mosaic and the wheat curl mite. NSW Department of Primary Industries Primefact 99.

Newton, A. C., Duncan, J. M., Augustin, N. H., Guy, D. C., and Cooke, D. E. L. 2010. Survival, distribution and genetic variability of inoculum of the strawberry red core pathogen, *Phytophthora fragariae* var. *fragariae*, in soil. Plant Pathol. 59:472-479.

Niblett, C. L., Zagula, K. R., Calvert, L. A., Kendall, T. L., Stark, D. M., Smith, C. E., Beachy, R.N., and Lommel, S. A. 1991. cDNA cloning and nucleotide sequence of the *Wheat streak mosaic virus* capsid protein gene. J .Gen. Virol. 72:499-504.

Nilsson, H. E. 1995. Remote sensing and image analysis in plant pathology. Annu. Rev. Phytopathol.33:489-527.

Nomenclature Committee of the International Union of Biochemistry (NC-IUB). 1986. Nomenclature for incompletely specified bases in nucleic acid sequences: recommendations 1984. P Natl Acad Sci. 83:4-8.

Oerke, E.-C. 2006. Crop losses to pests. J Agr Sci. 144:31-43.

Ohshima, K., Yamaguchi, Y., Hirota, R., Hamamoto, T., Tomimura, K., Tan, Z., Sano, T., Azuhata, F., Walsh, J.A., Fletcher, J., Chen, J., Gera, A., and Gibbs, A. 2002. Molecular evolution of *Turnip mosaic virus*: evidence of host adaptation, genetic recombination and geographical spread. J. Gen. Virol. 83:1511-1521.

Picado, A., Speybroeck, N., Kivaria, F., Mosha, R. M., Sumaye, R. D., Casal, J., and Berkvens, D. 2011. Foot-and-Mouth Disease in Tanzania from 2001 to 2006. Transbound Emerg. Dis. 58:44-52.

Pozdnyakova, L., Oudemans, P. V., Hughes, M. G., and Gimenez, D. 2000. Estimation of spatial and spectral properties of phytophthora root rot and its effects on cranberry yield. Paper presented at the Geospatial information in agriculture and forestry. 2nd International Conference on Geospatial Information in Agriculture and Forestry, Florida, USA,

Rabenstein, F., Seifers, D. L., Schubert, J., French, R., and Stenger, D. C. 2002. Phylogenetic relationships, strain diversity, and biogeography of tritimoviruses. J. Gen. Virol. 83:895-906.

Restrepo, S., Valle, T. L., Duque, M. C., and Verdier, V. 1999. Assessing genetic variability among Brazilian strains of *Xanthomonas axonopodis* pv. *manihotis* through restriction fragment length polymorphism and amplified fragment length polymorphism analyses. Can. J. Microbiol. 45:754-763.

Rice, P., Longden, I., and Bleasby, A. 2000. EMBOSS: The European Molecular Biology Open Software Suite. Trends Genet. 16:276-277.

Saleh, A. A., Ahmed, H. U., Todd, T. C., Travers, S. E., Zeller, K. A., Leslie, J. F., and Garrett, K. A. 2010. Relatedness of *Macrophomina phaseolina* isolates from tallgrass prairie, maize, soybean and sorghum. Mol. Ecol. 19:79-91.

Shukla, D., Frenkel, M., and Ward, C. 1991. Structure and function of the potyvirus genome with special reference to the coat protein coding region. Can. J. Plant Path. 13:178-191.

Singru, R., Sivaramakrishnan, S., Thakur, R. P., Gupta, V. S., and Ranjekar, P. K. 2003. Detection of genetic variability in pearl millet downy mildew (*Sclerospora graminicola*) by AFLP. Biochem. Genet. 41:361-374.

Somsen, H. W., and Sill, W. H. J. 1970. The wheat curl mite, *Aceria tulipae* Keifer, in relation to epidemiology and control of wheat streak mosaic. Kansas State University Agricultural Experiment Station Research Publication 162.

Stack, J., Cardwell, K., Hammerschmidt, R., Byrne, J., Loria, R., Snover-Clift, K., Baldwin, W., Wisler, G., Beck, H., Bostock, R.M., Thomas, C.S., and Luke, E. 2006. The National Plant Diagnostic Network. Plant Dis. 90:(2), 128-136.

Stack, J., and Fletcher, J. 2007. Plant biosecurity infrastructure for disease surveillance and diagnostics. In I. o. Medicine (Ed.), Global infectious disease surveillance and detection: Assessing the challenges - finding the solutions (Workshop summary, pp. 95-106). Washington, D.C: The National Academies Press.

Stack, J., Hammerschmidt, R., Hudler, G., Luke, E., and Bostock, R. 2007. National Plant Diagnostic Network: a record of accomplishment. CSREES five-year review. Retrieved from http://www.npdn.org/webfm_send/2

Stenger, D. C., Hall, J. S., Choi, I.-R., and French, R. 1998. Phylogenetic relationships within the family *Potyviridae*: *Wheat streak mosaic virus* and *Brome streak mosaic virus* are not members of the genus *Rymovirus*. Phytopathology 88:782-787.

Stenger, D. C., Hein, G. L., Gildow, F. E., Horken, K. M., and French, R. 2005. Plant virus HC-Pro is a determinant of eriophyid mite transmission. J. Virol. 79:9054-9061.

Stenger, D. C., Seifers, D. L., and French, R. 2002. Patterns of polymorphism in *Wheat streak mosaic virus*: sequence space explored by a clade of closely related viral genotypes rivals that between the most divergent strains. Virology 302:58-70.

Strange, R. N., and Scott, P. R. 2005. Plant disease: a threat to global food security. Annu. Rev. Phytopathol. 43:83-116.

Tomitaka, Y., and Ohshima, K. 2006. A phylogeographical study of the *Turnip mosaic virus* population in East Asia reveals an 'emergent' lineage in Japan. Mol. Ecol. 15:4437-4457.

Vincelli, P., and Tisserat, N. 2008. Nucleic acid-based pathogen detection in applied plant pathology. Plant Dis. 92:660-669.

APPENDIX A

WSMV Factsheet for CBIIAT

Family *Potyviridae*, Genus *Tritimovirus*

Host: Wheat (*Triticum aestivum*)

Figure 1: Chlorotic streaks on leaf of WSMV-infected wheat. Photo taken by Stephanie Rogers

Alternate Hosts:

- oats (*Avena sativa*)
- barley (*Hordeum vulgare*)
- rye (*Secale cerale*)
- maize (*Zea mays*)
- sorghum (*Sorghum vulgare*)
- millets (*Panicum*, *Setaria*, and *Echinochloa* spp.)
- wild rice (*Zizania aquatica*)
- many wild grass species in the genera *Aeiglops*, *Agropyron*, *Bouteloua*, *Bromus*, *Cenchrus*, *Digitaria*, *Echinochloa*, *Elymus*, *Eragrostis*, *Haynaldia*, *Hordeum*, *Lolium*, *Panicum*, *Phalaris*, *Poa*, *Orizopsis*, *Setaria*, and *Stipa*

(6, 13, 15, 14, 2)

I. Geographic Distribution

WSMV has been found in Canada, the Czech Republic, Hungary, Iran, Jordan, Mexico, Poland, Romania, Russia, Turkey, Yugoslavia, Zambia, Australia, and the USA (1, 5, 11, 16, 17, 18, 19, 20, 21). Within the USA, the Great Plains region has been impacted the most by WSMV. The specific regions affected within each state or country may change in future years. The area extension specialist or diagnostic lab should be able to provide more specific information on prior infections.

Examples of Assessment:

- True: WSMV is found annually within a 100-mile radius from this location
- Partially True: WSMV has been found within a 100-mile radius from this location within the last 10 years but is not found every year
- False: WSMV has not been found within a 100-mile radius from this location within the last 10 years

II. Vector and Disease Transmission

The wheat curl mite (*Aceria tosichella*; formerly *Aceria tulipae*; Figure 2), an eriophyid mite, is the only known natural vector of WSMV (4, 21). The mite is microscopic, measuring only 250 µm in length, making it very hard to see with the unaided eye (12). It is cigar-shaped and yellow-white in color. The mite relies predominantly on the wind currents for movement, as it is wingless and has only 2 pairs of very small legs, but can be carried by aphids and thrips (12, 22).

A mite has 4 life stages: egg, two nymphal stages, and adult. The mite passes through all 4 stages in 7 – 10 days. The nymph is the only life stage in which the mite can acquire the virus. After acquisition, either the nymph or the adult may transmit the virus (4, 22).

Mites require a green host for feeding and reproduction. Mite feeding on wheat causes curling of the leaves, protecting the mite from environmental conditions (24). Within the rolled leaves, the mites can survive for months. The mite overwinters on winter wheat, where it can survive for several months at near-freezing temperatures. When the winter wheat matures and is no longer suitable for feeding, the mite is dispersed by the wind onto its summer host, which may be volunteer wheat, grasses, or summer crops (24).

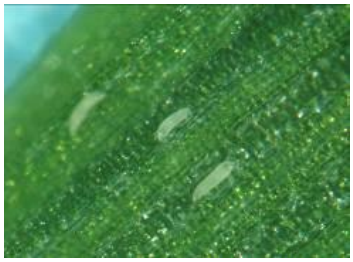

Figure 2: Close-up of wheat curl mites on a wheat leaf. Photo taken by Don and Chanda Heene of Texas AgriLife Research

Examples of Assessment:

- True: The wheat curl mite is present in the field or has been seen in the past few months; there is evidence of the mite, such as leaf curling/rolling
- Partially True: The wheat curl mite has been noticed in the area in the recent past (within 5 years)
- False: Wheat curl mite is not present and there is no evidence of the mite; mite or mite damage was never noticed in the field or surrounding areas in the past.

III. Spatial Distribution

The only natural inoculation of the virus is through feeding of the wheat curl mite on plant material. Since both the virus and the mite require a green host to live, the presence of volunteer wheat or alternate hosts, known as a "green bridge," is required for survival during the time between harvest and planting (24). The location of the green bridge is important in

determining if the pattern is unusual or not. Typically, volunteer wheat will be found along the edge of the field, but it may also be dispersed with in the field, especially if the field was not tilled after the previous year's harvest.

Figure 3: View west of a wheat field infected with WSMV. Note the yellow gradient of the leaves as it moves into the center of the field. Photo taken by Stephanie Rogers.

The distribution of the virus in a field and other surrounding areas will generally correlate with the wind direction because of the mites' dependence on the wind for dissemination (figure 3; 22). The distance into the field interior that contains virus infected plants will also correlate with the wind speed. The stronger the wind, the farther the mite will travel, and the larger the infected area. The proportion of infected plants is typically much greater near the source of inoculum than elsewhere (22).

Examples of Assessment:

- True: The virus obviously originated from volunteer wheat along the fence row; virus spread correlates well with the wind direction during time of mite activity/inoculation;
- Partially True: Wind direction does not obviously correlate with the spread of the virus; the general site of origin of the virus is not distinguishable, but is speculated to be volunteer wheat or alternate host;
- False: The spread of the virus is in the opposite direction of the wind; no, or very little, spread of the virus is observed.

IV. Favorable Weather

The ability of the mite and the virus to survive in the environment is important to determine whether the introduction was natural or intentional. The mite is the only natural means

of infection (except for extremely rare transmission by seed). Therefore, if the mite did not survive local weather, the virus could not have spread.

Mites tend to remain inactive during cold months of winter, but become active again after a few warm days (24). In a greenhouse study, mites survived temperatures as low as -3° C (26.6° F), but they were at that temperature for only 2.5 minutes. When tested for endurance to the cold for up to 16 days, survival occurred only as low as -5° C (23° F). Mites also survive summer temperatures as high as 50° C (122° F) (22).

Mites demonstrated normal population growth in 100% humidity at temperatures of 5°, 15°, and 25° C (41°, 59°, 77° F, respectively). At 25% relative humidity, no mites survived (22). The mite population increases rapidly at 24° C (75.2° F) or higher. Frequent rains and cooler-than-average summer temperatures also favor mite increase and survival (24).

Examples of Assessment:

True: Temperatures during the winter did not drop below freezing for more than a few days; relative humidity was greater than 25% when the temperatures were 25° C (77° F) or higher;

Partially True: The conditions of the winter months were suitable for mite survival, but the spring and summer months were not as favorable, perhaps having low humidity and/or high temperatures;

False: The winter months were extremely cold (-5° C/22° F or lower for several days) and/or there was a drought in the spring and summer. The mite definitely could not survive.

V. Temporal

Winter wheat, the predominant type of wheat grown in the southern U.S., is planted in the fall (typically late August to November) and harvested in the spring (typically late May or June). Spring wheat, grown predominantly in the northern U.S. and in Canada, Europe, and Asia, is planted in the spring and harvested in the fall.

When wheat becomes infected with WSMV, symptoms indicate disease incidence. Symptom development and severity of wheat streak mosaic are dependent on the time of planting and infection. Viruliferous mites may infect winter wheat with WSMV in either the fall or spring, but symptoms usually do not appear until the spring and become more severe as the temperature rises (9). Wheat planted and infected in the fall shows a greater disease incidence and severity than wheat planted in the fall and inoculated in the spring. Wheat planted in late fall (November) and inoculated in the spring shows significantly greater disease severity than wheat planted early in the fall (September or October) and inoculated in spring (9). These findings illustrate the dependence of severity on the maturity of the wheat at the time of infection.

Examples of Assessment:

True: First symptoms appear in early spring when temperatures start rising (for winter wheat); severity of disease is typical;

Partially True: First symptoms appear earlier than normal, e.g. in January, possibly influenced by an unusually warm winter; symptoms appear at a typical time but are significantly more severe than usual;

False: First symptoms appear in late fall/early winter; or symptoms appear in the fall and are significantly more severe than is typically expected.

VI. Field History

The history of the field can be important in identifying an intentional introduction. The virus can reoccur year after year in a location if a green bridge is present. It is important to determine whether the virus was present within the past 5 years.

How the field was treated before, during, and after harvest also is important. For instance, if the farmer does not cultivate the ground after harvest it is more likely that volunteer wheat will be present during the off-season, serving as a green bridge (23). Also, drought during the summer and fall will mean that wheat seed left in the field will not germinate. The farmer might have applied a herbicide, such as Round-up, to eliminate weeds that could serve as green bridges. Without a green bridge, neither the virus nor the mite will survive, and the current year's disease would be due to a new introduction that might or might not have been intentional.

Examples of Assessment:

- True: WSMV was found in the field the previous year or within the last 5 years; the field was not cultivated after harvest or before planting; the field was not treated with an herbicide to eliminate potential volunteer host plants;
- Partially True: No apparent infection occurred within the past 5 years; however, latent disease might have occurred (there was suspicion of disease but no confirmation was done); the field was cultivated after harvest but not before planting;
- False: No WSMV has ever been found in the field; the field was cultivated after harvest and before planting; the field was treated with chemicals to remove volunteers.

VII. Surrounding Areas

The mite's ability to move via the wind allows for a large area to become infected. If WSMV is present in a nearby field or grassy area, and if wind speed and direction were suitable for mite dispersal, the virus may be present in the field. If the field, or area, has never been infected with WSMV before, natural introduction may be due to wind dispersal of viruliferous mites into the field from a nearby-infected area. Once the virus has established itself in the field, it may persist for years if the appropriate green material is available year round and mites are present to continue virus spread. For winter wheat, the wind direction changes with the seasons, but for spring wheat, the wind direction remains relatively more constant throughout the growing season. If areas upwind of the field (taking into account the change in wind direction for winter wheat) are also infected with WSMV, that is a good indication of natural spread by viruliferous mites. If there are WSMV infections downwind, it is likely that mites carried the virus there from the field of interest, which may or may not indicate it is a natural occurrence.

Figure 4: View of southern boundary of WSMV-infected field. Notice the numerous grassy hosts along the fence line that may have served as hosts to the mite and virus. Photo taken by Stephanie Rogers

The presence of volunteer wheat, grasses, or alternative hosts, such as maize, is important to notice (Figure 4). As the wheat matures it dries out and the mites must move to a new host, or they will die. Winter wheat matures from May to June, and spring wheat matures from August to September. If a volunteer or alternate host is present at that time, the mite will reside and oversummer/overwinter there until the next wheat crop emerges (3). A control method used by some farmers to eliminate the green bridge created by volunteer wheat or by alternate weeds is to remove all potential viral hosts 3 weeks before planting. The mite is able to survive only 2 weeks without a green host, therefore eliminating the potential for viral spread from infected volunteer

plants (wheat or alternate hosts). Depending on the time of year the investigation is taking place, volunteer or alternate hosts may not be present. In that case, it is important to talk with a farmer or county agent who may have noticed whether such hosts were present and whether WSMV symptoms appeared.

Examples of Assessment:

- True: Volunteer wheat, grasses, alternate hosts, or nearby fields are also infected with WSMV or were infected at the time of planting; no removal of volunteer wheat or grasses prior to planting;
- Partially True: Potential plant hosts nearby are suspected to have been infected but disease was never confirmed;
- False: No infection is present in any surrounding green material and was never noticed; the infected field is the only infected area within 5 miles; all volunteer wheat or grasses were removed 3 weeks prior to planting; severe drought occurred in spring and fall (for winter wheat), preventing any volunteer hosts from germinating and growing.

VIII. Crop Rotation

As noted above, the green bridge is very important to the survival of both the virus and the mite. One way to eliminate this green bridge is to rotate wheat with another crop that is a non-host to the virus or mite. If there is no potential host available for the mite to live on between the wheat rotations, there should be no carryover virus present in the next wheat crop. It is important to remember there are many aspects to consider when determining whether a disease is a natural occurrence, and crop rotation does not provide absolute control.

Examples of Assessment:

- True: Wheat was not rotated with another crop, or was rotated with a host of the mite and/or virus, such as maize;
- False: Wheat was rotated with a non-host of the virus.

IX. Human Activity

Some farmers, extension agents, or other personnel may walk or drive farm machinery through the field periodically to inspect the condition of the wheat or to irrigate or apply chemicals to the field. These activities are normal. The farmer or extension agent should be contacted to determine the origin of any footprints or tire tracks noticed. If an intentional inoculation occurred in the field, the perpetrator may have been seen in the field. It is important to communicate with the farmer, neighbors and other persons to determine if unauthorized persons were present in the field or if unusual activity was noticed, such as a person lingering around the field or spraying an unknown substance on the field.

Examples of Assessment:

- True: No unusual activity, such as lingerers, unauthorized persons, or unfamiliar crop dusting was noticed or reported; all human activity noted can be accounted for by the farmer;
- Partially True: There is evidence/report of normal human activity but farmer was not active in the field; there may have been personnel but actions not accounted for; a car was seen parked near the field but no person was spotted;
- False: A person(s) was seen lingering in the field; an unauthorized person(s) was spraying an unknown substance on the wheat; unusual number of footprints noted, that cannot be accounted for by farmer.

X. Physical Evidence

In any crime scene, physical evidence is vital to the overall investigation and may help to place a person at the scene. Physical evidence may include footprints, tire tracks, trash, clothing, inoculation material, hair, and possibly even fingerprints or DNA. It is important to follow the standard forensic procedures for collecting such evidence to maintain its integrity. However, it is important to remember that legitimate farmers may leave evidence of their presence, and people driving by may throw trash out their window.

Examples of Assessment:

- True: No trash, prints, or other human physical evidence was found at the scene;
- Partially true: Some trash and footprints were found, but the farmer is most likely responsible;
- False: An inoculation device was found; footprints that do not match the farmer or other authorized people were found; rubber gloves or other possible inoculating materials were found.

XI. Motive

A key element in identifying a crime is to discover a motive. Motivations to commit a crop-related crime might be to spite one's boss after being laid off, or to eliminate market competition from a neighbor's crop. These types of motivation would characterize a biocrime: a crime committed by a person using a biological agent to harm another person or persons. Looking at a much larger scale brings us to bioterrorism or biowarfare, meaning an attack on the nation as a whole. This type of attack may be motivated by a hate for the government, a desire to take over the country, or the desire to endanger the economy.

Evidence of a biocrime may be a threat letter written by a disgruntled employee or someone with a grudge. If a farmer is envious of the success of another farmer, hateful words or threats may have been spoken to, or within hearing of, an outsider indicating a desire to sabotage a crop. Circumstances that could accompany a bioterrorism attack could include an ongoing war between countries; evidence could include a letter sent to the government indicating an intentional release of a pathogen, or the arrival in the U.S. of a particular individual who has carried out similar activities in other places. If multiple fields become diseased across the nation at the same time or in the same way, this could also be an indication of a national attack.

Examples of Assessment:

- True: No letters or threats have been noticed; no enemies or disgruntled persons are known;
- Partially True: Employees have been laid off or neighbor farmers have not been successful, but no threats are apparent;
- False: The farmer received a threatening letter; another farmer was overheard speaking about the plan of ruining another's crop; a war is ongoing between countries; multiple fields have arisen with commonalities causing suspicion of connection.

XII. Pathogen

Because of genetic drift and mutations caused by environmental stresses, multiple WSMV strains exist in each location and host. For instance, WSMV strains in the U.S. will differ from those in Australia. Virus strains also vary within the U.S., a diversity that allows the capability to define native strains, or strains that are common to particular areas.

It is important in every plant pathogen investigation to send several samples of infected material from several points in the field to a plant disease diagnostic lab to determine the presence and strain(s) of the pathogen. Identification of the strain as exotic or as one previously found

only in areas remote from this site may raise the question of an intentional introduction. However, the possibility exists that such a strain may have been seed-borne because WSMV is seed-borne in rare cases in wheat and maize (8, 10). It is advisable to have the seed lot tested for the presence of the virus.

Examples of Assessment:

- True: Viral strain(s) is normally found in the area; seeds from seed lot planted are infected with WSMV;
- Partially True: Strain(s) rarely found in the area; strain(s) not found in the area, but are native to a nearby region;
- False: The strain(s) has never been found in the area; the strain(s) is a foreign strain (from another country); the seeds are not infected with WSMV.

Glossary of terms as they relate to WSMV

Acquisition – receipt of a virus (pathogen) from an infected leaf by a vector (insect) when feeding.

Alternate Host – a plant that is not the preferred habitat or the host of concern for a pathogen or insect but still provides essential materials for pathogen/insect survival.

Area and County Extension Communicator or Agent – a professional located in a specific agricultural area or county who communicates with growers and researchers concerning their area of specialty. Extension communicators or agents may be hired at the city, county, regional, or state level.

Biocrime - a crime committed by a person using a biological agent to harm another person or group.

Biological Agent – a pathogen or other biological material that could be used to harm a person or group.

Bioterrorism – a crime committed by a person or group using a biological agent to harm a government or nation.

Decision Tool – a program or document designed to assist in decision making for designated situations.

Dissemination – the dispersal of a pathogen.

Exotic – not present in this country, but present in a foreign country.

Genetic Drift – fluctuations in the appearance of genes in populations due to random chance.

Great Plains Region of U.S.– central region of the United States, extending from Texas north to Montana, and from Colorado east to Iowa.

Green Bridge – plants that provide habitat and/or nutrition for insects and/or pathogens over a time when the primary host is absent. For WSMV, green bridge plants may include hosts such as grasses, summer crops, or volunteer wheat.

Grower/Producer - person responsible for the growth of crops.

Herbicide – a chemical, such as Round-up, that kills living plants.

Host – a living organism that provides habitat and nutrition for another organism. For WSMV, a host may be a plant (wheat or other grasses) or an insect (wheat curl mite).

Infection – virus (pathogen) is inoculated/transmitted to a plant, in which it colonizes, causing a disease.

Inoculation – act of pathogen introduction, mechanically or naturally, onto or into a plant host.

Inoculation Materials – items used to inoculate a plant with a pathogen; examples include a mortar and pestle or sprayer.

Inoculum – substrate or liquid containing the pathogen, used for inoculation; typically contains ground infected leaf tissue and a wounding agent, such as carborundum or celite, for mechanical inoculations.

Intentional Introduction – The act of purposefully introducing a plant pathogen into a crop or other plant environment.

Mutation – a change in the DNA/RNA sequence of a genome; often results in a new strain.

Natural Introduction – a pathogen being dispersed throughout crops via natural means, such as wind, rain, or insects.

Oversummer (used as a verb) – act of pathogen survival during the summer season by accessing a supportive niche on a plant or within a resting structure.

Overwinter (used as a verb) - act of pathogen survival during the winter season by accessing a supportive niche within a plant or developing a resting structure.

Plant Disease Diagnostician – a professional working in a plant disease diagnostic lab who analyzes plant samples for the presence of a pathogen.

Perpetrator – person who commits a crime.

Plant Pathogen – a biological agent (viruses, bacteria, fungi, nematodes, or parasitic plants) that can cause disease in a plant.

Severity – the level of infection within a plant; often measured by percentage of plant material showing symptoms.

Spring Wheat – a type of wheat that does not require a cold period (vernalization) to induce the reproductive phase of the life cycle. Spring wheat typically is planted in late spring/early summer and harvested in the fall; predominantly grown in Canada, Europe, and Asia.

Strain - pathogens of the same species that have distinct characteristics.

Symptom – specific physical appearances or physiological activities of a plant when diseased; some symptoms include chlorosis, necrosis, wilting, stunting, and lesions.

Transmission – act of the vector (often an insect) introducing a pathogen into a healthy plant.

Viruliferous – vector (insect) that contains a virus and is able to transmit that agent to a plant.

Volunteer Wheat – wheat that grows from grains dropped and left in a field after wheat harvest.

Winter Wheat – a type of wheat that requires a cold period (vernalization) to induce the reproductive phase of the life cycle. Winter wheat typically is planted in fall/early winter and harvested in late spring/early summer; predominant type grown in the U.S.

References

1. Ashworth, L. J. Jr., and Futrell, M. C. 1961. Sources, transmission, symptomatology, and distribution of *Wheat streak mosaic virus* in Texas. Plant Disease Reporter. 45:220-224

2. Berger, P. H., Percich, J. A., Ransom, J. K. 1981. *Wheat streak mosaic virus* in Wild Rice. Plant Dis. 65:695-696

3. Christian, M. L., and Willis, W. G. 1993. Survival of *Wheat streak mosaic virus* in grass hosts in Kansas from wheat harvest to fall wheat emergence. 77: 239-242

4. Del Rosario, M. S. E., and Sill, W. H. Jr. 1965. Physiological strains of *Aceria tulipae* and their relationships to the transmission of *Wheat streak mosaic virus*. Phytopathology. 55:1168-1175

5. Ellis, M. H., Rebetzke, G. J., and Chu, P. 2003. First report of *Wheat streak mosaic virus* in Austral. Plant Pathol. 52:808

6. French, R., and Stenger, D. C. 2002. *Wheat streak mosaic virus*. AAB Descriptions of Plant Viruses, NO. 393

7. Grunrow, R., and Finke, E. J. 2002. A procedure for differentiating between the intentional release of biological warfare agents and natural outbreaks of disease: its use in analyzing the tularemia outbreak in Kosovo in 1999 and 2000. Clin. Microbiol. Infec. Dis. 8:510-521

8. Hill, J. H., Martinson, C. A., and Russell, W. A. 1974. Seed transmission of Maize dwarf mosaic and Wheat streak mosaic viruses in maize and response of inbread lines. Crop Sci. 14:232-235

9. Hunger, R. M., Sherwood, J. L., Evans, C. K., and Montana, J. R. 1992. Effects of planting date and inoculation date on severity of Wheat streak mosaic in Hard Red Winter Wheat cultivars. Plant Dis. 76:1056-1060

10. Jones, R. A. C., Coutts, B. A., Mackie, A. E., and Dwyer, G. I. 2005. Seed transmission of *Wheat streak mosaic virus* shown unequivocally in wheat. Plant Dis. 89:1048-1050

11. Kapooria, R. G., and Ndunguru, J. 2004. Occurrence of viruses in irrigated wheat in Zambia. OEPP/EPPO Bulletin. 34:413-419

12. Keifer, H. H. 1938. Eriophyid studies. Department of Agriculture Bulletin. pp 181-206

13. McKinney, H. H. 1949. Tests of varieties of wheat, barley, oats and corn for reaction to *Wheat streak mosaic virus*. Plant Disease Reporter. 33:359-369

14. McKinney, H. H., and Fellows, H. 1951. Wild and forage grasses found to be susceptible to the *Wheat streak mosaic virus*. Plant Disease Reporter. 35:441-442

15. McKinney, H. H. and Sando, W. J. 1951. Susceptibility and resistance to the *Wheat streak-mosaic virus* in the Genera Triticum, Agropyron, Secale, and certain hybrids. Plant Disease Reporter. 35:476-479

16. McMullen, M. P., and Nelson, D. R. 1989. Wheat streak mosaic severe in 1988. North Dakota Farm Research. 46:14-16

17. Orlob, G. B. 1966. Epidemiology of Wheat streak mosaic in South Dakota, 1962-1966: Host range studies. Plant Disease Reporter. 50:819-821

18. Rabenstein, F., Seifers, D. L., Schubert, J., French, R., and Stenger, D. C. 2002. Phylogenetic relationships, strain diversity and biogeography of tritimoviruses. J. Gen. Virol. 83:895 - 906.

19. Sanchez-Sanchez, H., Henry, M., and Alvizo-Villasana, H. F. 2001. Identification of *Wheat streak mosaic virus* and its vector *Aceria Tosichella* in Mexico. Plant Dis. 85:13-17

20. Shahwan, L. M., and Hill, J. P. 1984. Identification and occurrence of *Wheat streak mosaic virus* in winter wheat in Colorado and its effects on several wheat cultivars. 68:579-581

21. Slykhuis, J. T. 1953. Wheat streak mosaic in Alberta and factors related to its spread. Can. J. Agricul. Sci. 33:195-197

22. Slykhuis, J. T. 1955. *Aceria tulipae* Keifer (Acarina: Eriophyidae) in relation to the spread of Wheat streak mosaic. Phytopathology. 45:116-128

23. Slykhuis, J. T., Andrews, J. E., and Pittman, U. J. 1957. Relation of date of seeding winter wheat in Southern Alberta to losses From Wheat streak mosaic, root rot, and rust. Can. J. Plant Sci. 37:113-127

24. Somsen, H.W., and Sill, W.H. Jr. 1970. The Wheat Curl Mite, *Aceria tulipae* Keifer, in relation to epidemiology and control of Wheat streak mosaic. Research Publication Series of the Kansas Agricultural Experiment Station. Research Publication 162

APPPENDIX B

Plant Disease Worksheet

During a forensic investigation, thorough documentation of all aspects is critical for the evidence to be admissible in court. While documentation procedures are well developed for standard criminal investigation, the elements of a plant disease outbreak that should be documented remain unclear. This plant disease worksheet was designed to be a component of the Crop Bioagent Introduction Intent Assessment Tool (CBIIAT) (described in Chapter III) to assist investigators in properly documenting the disease outbreak in its entirety. The format of the worksheet is fashioned from the sample submission form for the Oklahoma State University Plant Disease and Insect Diagnostic Laboratory, which can be found online at http://entoplp.okstate.edu/pddl/pdidl-form.pdf. The worksheet is available in downloadable form from the CBIIAT website (www.bioinfosu.okstate.edu/NIMFFAB).

Investigating Agency:_____ Case #:_____

Investigator(s): _____ Date: _____

Contacted by: _____

_____

Location:

State: _____ County: _____ Nearest Town: _____

GPS: _____

Driving Directions: _____

_____

Grower Information:

Name: _____ Spouses Name: _____

Address: _____

Work Phone: _____ Cell Phone: _____ Fax: _____

Email: _____

Time at Address:_____ Acres of Farming Land: _____ Farming Since: _____

Crops: _____

Extension Agent:

Name: _____Employer: _____

Address: _____

Work Phone: _____ Cell Phone: _____ Fax: _____

Email: _____

Area of Expertise: _____ Years as Agent: _____

Plant Disease:

Host Plant(s) and Varieties: _____

Disease: _____Pathogen: _____

Plant Parts Affected:	**Symptoms:**		**Distribution:**
☐ Roots	☐ Spot	☐ Dieback	☐ Single plant
☐ Stem	☐ Mottle	☐ Root rot	☐ Scattered plants
☐ Leaves/Needles	☐ Distortion	☐ Stem rot	☐ Group(s) of plants
☐ Twigs/Branches	☐ Yellowing	☐ Burn/scorch	☐ Entire planting
☐ Trunk	☐ Stunted	☐ Galls/swelling	☐ Low, moist areas
☐ Buds	☐ Wilt	☐ Fruit rot	☐ High, dry areas
☐ Fruit	☐ Canker	☐ Other: _____	☐ Other: _____
☐ Flowers	☐ Shot hole		

Symptom Degree of Severity: ☐ low ☐ moderate ☐ severe

Severity of pathogen: (Check all that apply)

☐ Select Agent ☐ High Consequence Pathogen
☐ Low Consequence ☐ Native Species
☐ Annual Occurrence ☐ Former Select Agent

Field Information:

Total Acres: _____ Acres Affected: _____

Date Symptoms Appeared or noticed: _____Seed Came From: _____

Planting Date or Age of Plant: _____

Crop 1 year ago: _____ Crop 2 years ago: _____

Soil type: Sand(%)_____ Loam(%)_____ Clay(%)_____ Soil pH_____

Results of Soil Test: _____

Soil Treatments (what and when): _____

_____

_____

Fencing (check all that apply):

- ☐ Barbed/Barbless Wire
- ☐ Wood
- ☐ Cattle Panels
- ☐ Chicken Wire
- ☐ Metal
- ☐ No Fences
- ☐ Plastic
- ☐ Vinyl

Field Borders (check all that apply):

- ☐ Bare Soil
- ☐ Overgrown grass/weeds
- ☐ Cut grass/weeds
- ☐ Fungicide Applied (name:_____)
- ☐ Insecticide applied (name:_____)
- ☐ Herbicide applied (name:_____)
- ☐ Border Crop _____
- ☐ Other_____

Surrounding Areas:

Neighboring Fields:

Location to Field	Crop Planted	Present Diseases	Divider (i.e. fence)	Past Diseases

Nearby Water Source:

Type of Water Source	Proximity/Location to Field	Used for Irrigation?	Pathogens Present

Nearby Volunteer Plants:

Volunteer plants present: Yes ☐ No ☐

If yes, what plants: _____

Volunteer plants are potential hosts of pathogen: Yes ☐ No ☐

Volunteer plants are infected with same disease: Yes ☐ No ☐

Weather Information

Temperature during the month prior to symptom development:

☐ Hot (>90F) ☐ Warm (70F–89F) ☐ Cool (50F–69F) ☐ Cold (30F–49F) ☐ Freezing

Moisture during the month prior to symptom development:

☐ Excessive Moisture ☐ Adequate Moisture ☐ Heavy dews ☐ Drought

Wind speed up to a month prior to symptom development:

☐ No Wind ☐ Light Wind ☐ Moderate Wind ☐ Heavy Wind

Sky covering during the month prior to symptom development:

☐ No Clouds ☐ Few Clouds ☐ Partly Cloudy ☐ Mostly Cloudy ☐ Overcast

Weather conditions appropriate for vector and pathogen survival? Yes ☐ No ☐

Plant Condition Prior to Symptom Development

☐ Healthy ☐ Declining ☐ Damaged; If so, How? _____

Field Treatments

Chemicals Applied:

Name	Type	Dates Applied
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Equipment Cleaned between Uses? How? _____

_____

Field Preparation Prior to Sowing:

	Dates Performed (If applicable)
☐ Conventional Tillage	_____
☐ Minimal Tillage	_____
☐ No Till	_____
☐ Other:_____	_____

Pest Management Protocols Currently in Place (i.e. Crop Rotation, Intercropping, Pesticides, Biocontrol, etc…):

_____

Alternative or Alternate Host Present:

Name of Plant	Time at Location	Proximity to Infected Plant
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Employee History:

Name	Title	Contact Information	Positive Evaluation?	Recent Hire/Fire?

Any motives present to harm the grower or industry? If so, explain._____

_____

_____

Vector

Insects Present: **Vector of Pathogen?**

_____ ☐ Yes ☐ No

_____ ☐ Yes ☐ No

_____ ☐ Yes ☐ No

_____ ☐ Yes ☐ No

Nearest Occurrence of Same Disease:

Location	Severity	Proximity to Field

Field History:

Disease	Dates Affected	Action Taken
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Other Incidents in the Nation that are Identical to this One:

Location	Crop	Disease	Distance	Common Factor Among Incidents
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____

Incident Contact(s): _____

Laboratory Results

Evidence of genetic engineering? ☐ Yes ☐ No

Seed tested for pathogen? ☐ Yes ☐ No

If seed was tested, was pathogen present? ☐ Yes ☐ No

If pathogen was present, was pathogen an identical strain? ☐ Yes ☐ No

Causal Agent: _____

Strain (If Detected): _____

Origin (If Identified): _____

Procedures Used: _____

Date of Analysis: _____

Contact Information: _____

Physical Evidence

Item	Type of Evidence	Analysis Performed	Date

Miscellaneous Information

VITA

Stephanie Marie Rogers

Candidate for the Degree of

Doctor of Philosophy

Thesis: A DECISION TOOL AND MOLECULAR TYPING TECHNIQUE FOR A PLANT PATHOGEN FORENSIC APPLICATION, USING WHEAT STREAK MOSAIC VIRUS AS A MODEL PATHOGEN

Major Field: Plant Pathology

Biographical:

Personal Data: Born in Stillwater, OK on July 21, 1985, the daughter of Glen Alan Shryock and Paula Annette Shryock

Education: Received Bachelor of Science in Biochemistry and Molecular Biology at Oklahoma State University, Stillwater, Oklahoma in Dec, 2006; completed the requirements for the Doctor of Philosophy in Plant Pathology at Oklahoma State University, Stillwater, Oklahoma in May, 2011.

Experience: Research Assistant at Oklahoma State University from October 2005 to December 2006; Laboratory Technician at Accurate Environmental Laboratories, from January 2007 to June 2007; ORISE Visiting Scientist at Federal Bureau of Investigation Laboratory from May 2008 to August 2008; Graduate Research Assistant at Oklahoma State University from June 2007 to May 2011.

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Name: Stephanie Marie Rogers Date of Degree: May, 2011

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: A DECISION TOOL AND MOLECULAR TYPING TECHNIQUE FOR A PLANT PATHOGEN FORENSIC APPLICATION, USING WHEAT STREAK MOSAIC VIRUS AS A MODEL PATHOGEN

Pages in Study: 179 Candidate for the Degree of Doctor of Philosophy

Major Field: Plant Pathology

Scope and Method of Study:

The vulnerabilities of US agriculture and the easy availability of naturally abundant plant pathogens create the threat of a biological attack on our cropping systems. Before a forensic investigation of a disease outbreak occurs, it must be determined that the introduction was likely to have been intentional. Successful attribution of the crime may be facilitated by a genetic analysis of the causal pathogen. Strict method validations must be conducted for the procedure to withstand the scrutiny of the courtroom. In this study, I developed a decision tool to assist first responders in assessing whether an outbreak may have involved human intent. Additionally, I validated a molecular typing technique using *Wheat streak mosaic virus* (WSMV) as a model and evaluated its use in a forensic investigation through field trials and isolate relationship studies.

Findings and Conclusions:

The Crop Bioagent Introduction Intent Assessment Tool (CBIIAT) proved to be an effective method for assessing the likelihood that a plant disease outbreak was the result of a deliberate effort. The tool's effectiveness was evaluated by several "testers," individuals who practiced using the tool at both a natural and intentionally inoculated WSMV outbreak in Oklahoma. With easy access via the online program and a user-friendly design, the tool can be quickly incorporated into investigative procedures by non-scientists and used as a model for similar tools for other pathogens.

The thorough validation showed the WSMV SNP typing method to be reproducible, sensitive, specific, and capable of discriminating between known strains and field isolates of the virus. In a two year study I evaluated the effect of common wheat field treatments and conditions on the consistency of the single nucleotide polymorphism (SNP) profiles of WSMV isolates. No effects due to treatment were found. Minor changes found in the profiles were most likely a natural phenomenon.

The SNP profiles were generated in 2008 for two geographically distinct WSMV outbreaks to determine if the SNP typing method could be used to evaluate the pattern or pathway of spatial spread of the virus. No correlation of genotype to geographical location was found, but each isolate yielded a unique SNP profile which could be useful in a forensic investigation, particularly if a correlation were shown with another element, such as the origin of seed.

ADVISER'S APPROVAL: Jacqueline Fletcher