VALIDATING FORENSIC TOOLS FOR CROP BIOSECURITY: CASE STUDY INVESTIGATION OF SALMON BLOTCH OF ONIONS IN ISRAEL

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VALIDATING FORENSIC TOOLS FOR CROP BIOSECURITY: CASE STUDY INVESTIGATION OF SALMON BLOTCH OF ONIONS IN ISRAEL

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Title of Study: VALIDATING FORENSIC TOOLS FOR CROP BIOSECURITY: CASE STUDY INVESTIGATION OF SALMON BLOTCH OF ONIONS IN ISRAEL

Major Field: Plant Pathology

Globalization of agricultural commerce increases the vulnerability of the United States to introductions of plant pathogens by inadvertent or intentional means. Plant pathogen forensics combines traditional plant pathology and microbial forensics to enhance crop biosecurity. This research was designed to test and validate microbial forensic tools for plant pathogens in laboratory and field settings. A real-time PCR assay developed by the National Bioforensic Analysis Center for high consequence human pathogens was adapted and validated for the phytopathogenic bacterium, *Xylella fastidiosa*, which affects many plant species. PCR primers amplified genomic DNA from multiple strains of the bacterium and did not amplify near-neighbor microorganisms or animal or plant DNA. Other forensic tools were developed to investigate an actual outbreak, in Israel, of salmon blotch disease of onions, caused by the phytopathogenic fungus *Fusarium proliferatum*. A decision tool designed to assist first responders recognize signs of criminal activity at the field was implemented and a DNA fingerprinting assay using simple sequence repeats (SSRs) to discriminate among different pathogen populations was validated. *F. proliferatum* was isolated from onion and soil samples from the affected field, nearby agricultural fields and natural vegetation in southern Israel onion production areas. Fungal isolates were obtained also from onion sets (grown in northern Israel and shipped for planting in southern fields), to test a hypothesis that the fungus was disseminated on these sets. SSR analyses revealed that fungal populations from onion sets in northern Israel are genetically distinct from those in southern Israel. *F. proliferatum* populations from southern field site soils are similar to one another and to those from bulbs at each of four southern fields. By SSR analysis, *F. proliferatum* isolates from volunteer salt cedars in the onion fields are clonal and indistinguishable from those from the southern field soil and white onion bulbs. The findings suggest that onion sets purchased from northern Israel are not the source of the *F. proliferatum* causing onion salmon blotch in southern Israel. Furthermore, volunteer weeds, including salt cedar, and previously contaminated field soil could serve as alternative reservoirs for the fungus, from which inoculum could have moved to the onions

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CHAPTER I

Introduction

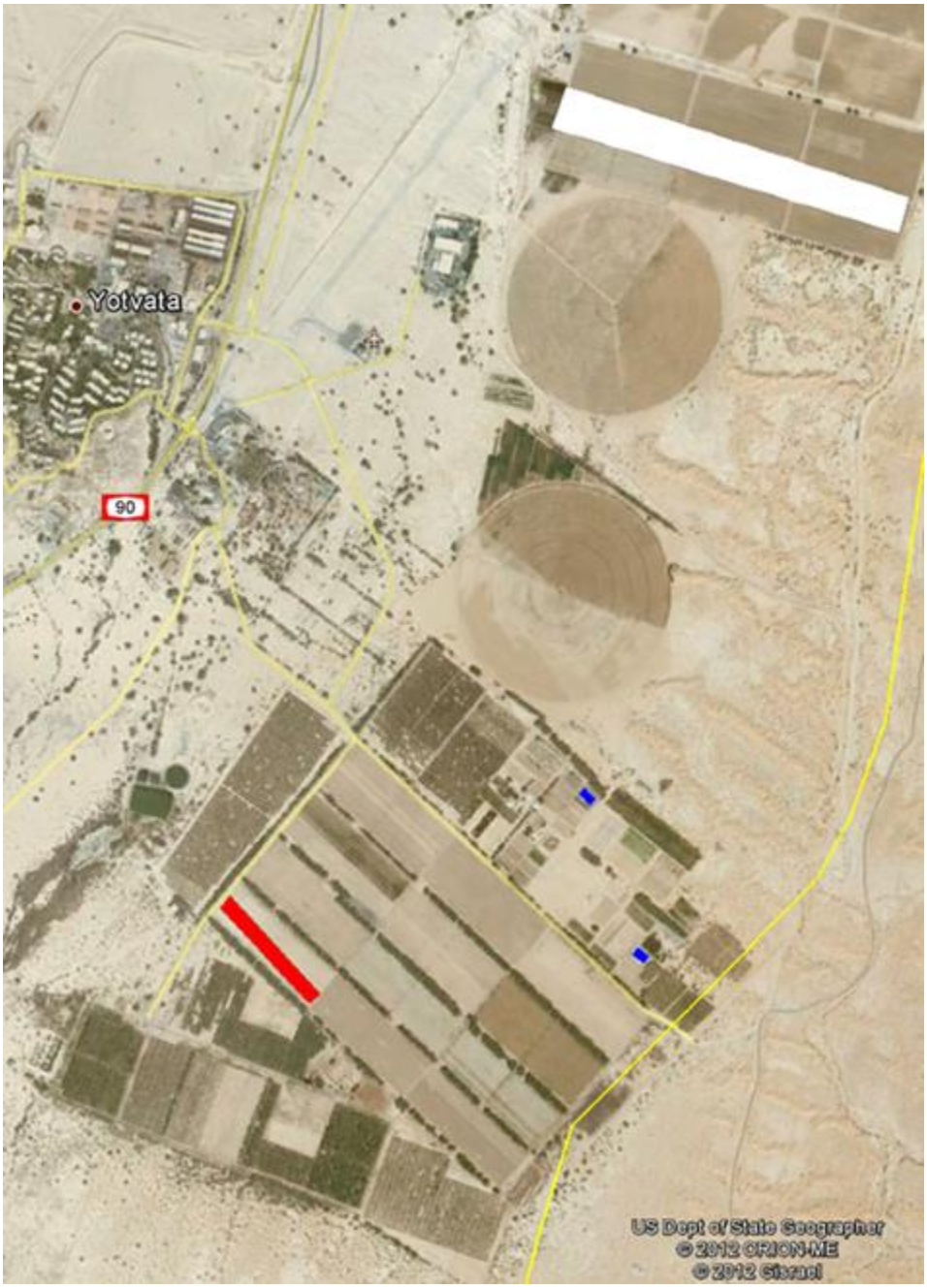
Salmon blotch of onion, caused by the fungus *Fusarium proliferatum*, was observed in southern Israel in 2012. The disease is characterized by salmon-colored blotches on the outer scales of white onion cultivars but the fungus also can be isolated from yellow and red onion cultivars. Onion production in Israel occurs in both northern and in southern Israel. Onion seeds, which are either imported or produced within Israel, are planted in northern Israel around the third week of January. Once the seeds germinate and produce small bulbs (sets) around mid-February, they are harvested and stored in sheds until they are sold to production farms in southern Israel. The sets are planted directly in the soil toward the end of August or early September and grow into mature bulbs, which are harvested in January or February before being sent to the local packing houses and sold. Onion sets used in this study, produced in Beit She'an (northern Israel), were planted in fields near the kibbutz towns of Yotvata and Grofit (southern Israel) (Figure 1). Plant and soil materials were collected from four fields near Yotvata, including two commercial fields (red and white rectangles) and two research plots (two blue rectangles) (Figure 2). All four fields were planted with white onion sets (cv. Milky Way).

One of the commercial fields contained three additional onion cultivars, Ada and Gobi (both yellow onions), and Mata Hari (red onions).

The hypothesis of this research is that the onion sets are infested with *F. proliferatum* when they are planted into the soils in the south, and that they are the source of the isolates causing the salmon blotch outbreak in 2012, in Yotvata, Israel.

Figure 1. Overhead view of Israel and the towns Beit She'an and Yotvata.

Figure 2. Four field sites used in this study. Two commercial fields (red and white large rectangles) and two research fields (blue rectangles).



Another component to this dissertation was to develop a real-time PCR assay for the detection of the bacterium *Xylella fastidiosa*, which is a pathogen to many grapevines, tree, and shrubs. The National Bioforensic Analysis Center has validated real-time PCR assays for many human and animal pathogens with stringent standards for forensic purposes. Our goal was to adapt the already established forensic assays for plant pathogens common to Oklahoma with hopes of using these assays for high consequence plant pathogens in the near future.

Objectives of the research

The overall goal of this research is to apply and validate forensic tools to investigate a 'real world' disease situation. The first objective was to validate a decision tool that will be used to help determine if the 2012 salmon blotch outbreak of onions in southern Israel, caused by *F. proliferatum,* was due to natural causes or due to nefarious actions. The second objective was to validate a DNA fingerprinting assay using simple sequence repeats (SSRs) to characterize isolates for *F. proliferatum* from various plant and soil sources, and the third objective was to apply the validated assay to a forensic investigation determine the source of the 2012 salmon blotch fungus.

CHAPTER II

Literature Review

I. Agroterrorism

Agroterrorism is the deliberate introduction of chemical, biological, or toxin based weapons against livestock and/or crops to threaten a nation's food supply and undermine government agencies (Carabin et al. 2005). Other possible targets include wildlife, forests, and rangelands. The use of biological and chemical weapons to contaminate water and food supplies is not a new concept. In fact, Greek, Roman, Persian, and Chinese literature depicts contaminating water sources with dead animal carcasses over 2,000 years ago. Bioterrorism was even demonstrated during the United States Civil War (1861-1865) in which the Confederate forces retreated and left dead and decaying animals behind to contaminate water sources for the Union Army (Carabin et al. 2005). World War I was the first event in the modern microbiological era in which anti-animal warfare occurred. To help their allies, the United States shipped cattle to Europe to supplement food supplies for the troops during the war (Carabin et al. 2005). In 1915, the Germans were accused of using *Bacillus anthracis* and *Burkholderia mallei* to infect cattle, horses and mules throughout Europe (Harris and Paxman, 2002). German infiltrators tried to develop large quantities of these pathogens in laboratories in the

United States, France, Romania, and Mesopotamia (Robertson and Robertson, 1995). Biological weapons were still being developed by Germany after World War II and by the former Soviet Union before the Cold War. Other countries which have developed biological weapons includes, Japan, France, Canada, United Kingdom, and the United States of America (Fletcher et al. 2006).

Although there are no documented cases of such an event in the United States, the agricultural sector provides terrorists with ample targets. There are several reasons why U.S. agriculture is vulnerable. Crops are often grown over large areas and it is almost impossible to monitor every part of a field in a "perfect" military sense (Madden and Wheelis, 2003). For example, in 2004 the total land area devoted to corn and soybeans was 81 million and 74.8 million acres, respectively (Nutter and Madden, 2005). As a consequence, a new disease may not be detected until after several generations of the pathogen are produced in the field (Madden and Wheelis, 2003). For example, it was not until 2.5 years after the natural introduction of *Xanthomonas citri* that citrus canker was detected by Schubert et al. (2001). Another vulnerability of U.S. agriculture is the country's long borders shared with Mexico and Canada. Port inspectors remind the public about the dangers of importing unapproved or uninspected products, but bioterrorists would be unlikely to declare agricultural products and could easily smuggle in tiny amounts of inoculum (Nutter and Madden, 2005). An agricultural attack by a bioterrorist may not do physical harm to a society but rather his/her motivation would be more of a political statement or cause economic distress to a country.

The introduction of a pathogen into a country is often times inadvertent, on shoes or clothing, trade commodities, migrating wildlife, and other moving entities. Another

factor is natural weather phenomena such as hurricanes, tsunamis, and dust storms. In November 2004, the United States Department of Agriculture (USDA) published a news release confirming the first case of Asian soybean rust, caused by the fungus *Phakopsora pachyrhizi*, at a Louisiana State University research farm (Release No. 0498.04). The USDA Animal and Plant Health Inspection Service (APHIS) believed that the active hurricane season the previous year was correlated to the occurrence of soybean rust in Louisiana.

Plant pathogens as biological weapons could be very attractive to a bioterrorist. One attractive aspect is that they are not harmful to the handler depending on which agent in being used. Only if they have severely compromised immune systems are humans susceptible to harmful effects of plant pathogens. A bioterrorist would not have to follow special laboratory procedures for the collection, storage, propagation, and dissemination of the pathogen (Nutter and Madden, 2005). Further, there are a multitude to choose from, the most prominent being fungi, bacteria, and viruses. There are more than 10,000 species of fungi, 100 species of bacteria, and 1000 viruses that attack plants (Agrios, 1997). However, in a specific region there are generally 5-20 devastating plant pathogens of a given plant species that cause severe economic loss on an annual basis. In the U.S. most crop species were established from other parts of the world and it is possible that the pathogens followed (Madden and Wheelis, 2003). Because it can take several weeks before a plant disease is detected, the pathogen and disease could be well established before being noticed. In addition, investigators would have to determine if the disease was intentional, accidental or due to environmental circumstances.

The social and economic impact of a biological attack against U.S. agriculture has the potential to be catastrophic. The most critical and damaging impact would be a ban placed upon imports of plant materials from the U.S. by members of the World Trade Organization (Nutter and Madden, 2005); resulting trade losses could reach millions to billions of dollars. An example of a pathogen introduction that led to significant impact on the United States' economy is the fungus *Tilletia indica*, causal agent of Karnal bunt in wheat. In 1996, Karnal bunt was discovered in Arizona on a single durum wheat kernel (Ykema et al. 1996). Karnal bunt was later detected on wheat in California, and infected seed was shipped to New Mexico, and Texas (Rush et al. 2005). The disease threatened U.S. agriculture because 50% of all U.S. wheat produced is exported. As a result, the Animal and Plant Health Inspection Service (APHIS), quarantined the entire state of Arizona and several fields in California, New Mexico and Texas. Between 1996 and 1998, APHIS spent over $60 million to try to eradicate the fungus, and during that time it was estimated that growers lost over $100 million in farm sales (Bandyopadhyay et al. 1999). Costs of plant pathogen containment can be as substantial as losses from reduced international trade. After citrus canker, caused by the bacterium *Xanthomonas axonopodis* pv. *citri*, arrived in Florida in 1994, the Federal government spent $100 million annually to try to eradicate the bacterium until the effort was stopped in 2006. In the end, the total cost of the eradication effort approached $1 billion, with annual losses suffered by the citrus industry of around 8 to 9 billion dollars per year (Bandyopadhyay et al. 1999).

Not only can a country's economy be negatively affected from a successful agricultural attack, but social unrest among the society could ensue (Casagrande, 2000).

If reports surfaced that certain types of food were tainted with a pathogen, then consumers would most likely not buy those product. If a single crop in a wealthy nation were to be largely lost for consumption, then people would shift to another food source. In less developed countries like the Philippines that rely heavily on rice as a part of the diet, an intentional or natural outbreak of a disease could lead to famine and political disruption (Fletcher et al. 2006). Perhaps the most significant effects a plant pathogen can have on a society occurred during the Irish potato famine (1845-1847). Late blight of potato, caused by the oomycete *Phytopthora infestans*, was responsible for 1 million deaths and the emigration of a 1.5 million Irish (Carabin et al. 2005).

Another example of the impact an agriculturally associated disease can have on a country was the foot-and-mouth disease outbreak in the United Kingdom in 2001. The socio-economic effect of this natural occurrence was profound. After its diagnosis in February 2001, 6 million animals, including 4.9 million sheep, 700,000 cattle, 400,000 pigs, 2,000 goats, and 1,000 deer, were destroyed (Carillo and Rock, 2005). Not only was the agricultural sector negatively affected, but tourism-related industries also experienced economic loss. The Department for Culture, Media, and Sport and the Department for Environment Food and Rural Affairs (DEFRA) in the UK estimated the impact on tourism due to FMD to be between £4.5 and £5.4 billion (US $3.9 to 4.6 billion)

"The Public Health Security and Bioterrorism Preparedness and Response Act of 2002 (Public Law 107-188; June 12, 2002) requires the United States to improve its ability to prevent, prepare for, and respond to acts of bioterrorism and other public health emergencies that may threaten public health and American agriculture"

(http://www.aphis.usda.gov/programs/ag_selectagent/). High risk plant pathogens are listed as select agents under the Code of Federal Regulations, title 7, part 331 and the complete list of select agents can be found at the USDA APHIS website (http://www.aphis.usda.gov/programs/ag_selectagent/ag_bioterr_toxinlist.shtml). The criteria for a pathogen to be put on the select agent list include, but are not limited to: a) the effect of an agent or toxin on animal or plant health or products, b) the virulence or degree of toxicity of the agent and the methods by which the agent or toxin is spread, and c) the availability of and effectiveness of medicines and vaccines to treat and prevent any illness caused by an agent or toxin (http://www.aphis.usda.gov/programs/ag_selectagent/ag_bioterr_QA.shtml). Strict regulations, such as registration, restrictions, and security measures that are required for the handling and investigation, are in place. These restrictions can help in determining attribution of a crime involving a select agent; however, if a plant pathogen on the select agent list is discovered in the U.S. and seems to be well established it may be delisted from the select agent list (Fletcher et al. 2006).

United States infrastructure and vulnerabilities in agricultural biosecurity

Fifty percent of all land in the United States is devoted to agriculture (486 million hectare); 186 million hectare and 284 million hectare as crop land and forestland, respectively (Fletcher and Stack, 2007). Agriculture is vital, and the U.S. has prepared for biological attacks against this sector by developing and deploying monitoring and detection systems. However, the events of September 11, 2001, on the World Trade Center and Pentagon, and then later, anthrax attacks on members of the U.S. Congress, raised awareness that the U.S. is vulnerable to such attacks and the agriculture sector too

is at risk (Sherwood et al. 2003). One vulnerability is the long borders that the U.S. shares with Mexico and Canada (Nutter and Madden, 2005). There are 126 legal points of entry around the U.S. (Sherwood et al. 2003) for agricultural products, and port inspectors can be the first line of defense in detecting illegal plant material. One way to better fortify U.S. agriculture against a biological attack would be to raise awareness of potential pathogens that could be of high consequence if introduced into the U.S. The American Phytopathological Society's (APS) Ad Hoc Emerging Diseases and Pathogens Committee is doing exactly that (Sherwood et al. 2003). It is important not only to predict what exotic pathogens will appear within U.S. borders and when, but to prepare plant diagnostic labs to identify the pathogen accurately and report to the proper authorities.

The National Plant Diagnostic Network (NPDN) (http://npdn.ppath.cornell.edu) was established to coordinate state, regional, and diagnostic laboratories to promote effective communication and a standard reporting process for plant pathogen identification (Sherwood et al. 2003). The NPDN is effective and serves as a cornerstone to the U.S. crop biosecurity infrastructure, but it is not without some limitations. During an investigation of a disease outbreak, many personnel, including first responders, may not be familiar with identifying plant diseases or insect vectors. The NPDN has an excellent training program for diagnosticians, but training for non-professionals is needed. Methods to track disease outbreaks in real-time are needed to help predict where the pathogen may spread (Sherwood et al. 2003). Perhaps the most difficult limitation to overcome is the lack of knowledge of many exotic plant pathogens because there are so many (Nutter and Madden, 2005). These limitations can be overcome with increases in

federal funding, collaborations between institutions, and stronger communication between the government, industry and stakeholders (Sherwood et al. 2003).

II. Microbial forensics

Microbial forensics is a scientific discipline devoted to analyzing evidence from a bioterrorist act, biocrime, or inadvertent release of a microorganism/toxin for attribution purposes (Breeze et al. 2005). What separates microbial forensics from other science disciplines is the process of attribution, which is the linking a pathogen and/or a perpetrator to a specific biocrime or bioterrorst act. Attribution includes identifying the pathogen(s) involved in the criminal act (Breeze et al. 2005) and identifying the person or people responsible for the criminal act. The components of microbial forensics described by Breeze et al. can be incorporated into plant pathogen forensics programs:

1) **Detection and identification of a pathogen** is an important part of a forensic investigation. Sensitivity and specificity of molecular assays must be validated and DNA-based systems and analytical chemistry methods may be modified depending on what questions are being asked during the investigation. Other techniques to identify and detect pathogens can include physical chemistry, tissue collection, and bioassays in animals.

2) **Genetic** i**nformation and DNA databases** are already being used in law enforcement for example, the Federal Bureau of Investigation (FBI) Combined DNA Index System (CODIS) and are used by local, state, and federal crime laboratories in the U.S. (http://www.fbi.gov/about-us/lab/biometric-analysis/codis). The CODIS database, which stores human DNA information, used by law enforcement to establish that a

suspect has been at a crime scene is commonly used in forensic investigations where genetic information is necessary to link a suspect to a crime scene. There are no databases like CODIS for microbes, including plant pathogens, but the Centers for Disease Control and Prevention (CDC) maintains PulseNet, which stores 'DNA fingerprints' of foodborne human pathogenic bacteria collected during outbreaks of foodborne illness (http://www.cdc.gov/pulsenet/). A broader public database containing DNA sequence information for numerous prokaryotic and eukaryotic organisms is the National Center for Biotechnology Information's (NCBI) GenBank (https://www.ncbi.nlm.nih.gov/genbank/), which includes sequences from international databases including the DNA DataBank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp/) and the European Molecular Biology Laboratory (EMLB) (http://www.ebi.ac.uk/). One drawback of such a large, publically available database is that anyone can input DNA sequences and sequence mistakes or mislabeling could go unnoticed for a long time.

3) **A strain repository** for pathogens and near-neighbor microorganisms is needed. The strains housed in the repository must be of high quality and well characterized so that they can serve as reliable reference material. Currently, plant pathologists can order certain isolates of bacteria, fungi, and viruses from companies such as the American Type Culture Collection (ATCC) and Agdia, however; not all species may be available. Generally, plant pathologists ask each other if they have a particular 'type' strain that can be used as a reference material but the process can be time consuming, which could delay an investigation.

4) **Validation** of forensics procedures (i.e. sample handling/collection, interpretation of data) is essential so that evidence presented in a courtroom will be

admissible. If a new procedure is needed for sample analysis it must be validated and rigorously reviewed. Plant pathologists may have to adapt current bioforensic assays already developed by government agencies such as the National Bioforensics Analysis Center (NBFAC).

5) **Quality assurance (QA)** guidelines must be established in a microbial forensics program. Lab personnel and the lab itself must adhere to standard QA guidelines and biological safety protocols. For example, a lab should be organized to eliminate chances for sample cross contamination. Lab equipment should be maintained and calibrated as needed as well as clean. If a lab develops and adheres to strict standard operating procedures (SOPs), then evidence from that lab is likely to be adequately robust to be accepted in a court room.

Role of microbial forensics in plant biosecurity

As plant pathogen forensics continues to emerge as a discipline, the need for establishing standard crime scene practices and evidence handling is needed, and procedures must be adapted and validated for plant pathogens (Fletcher et al. 2006). Some methods for investigating a plant disease outbreak have been suggested (Nutter, Jr., et al. 2004) including documentation of the potential crime scene, sampling procedures (where to sample, how to sample), identification of strains, isolates, or races of the pathogen in question, and determining the source of the pathogen for aiding in attribution or exclusion. To integrate plant pathogen forensics into an agricultural biosecurity framework requires close relationships with federal agencies like USDA APHIS, and the Department of Homeland Security (DHS).

NBFAC and NIMFFAB

The Battelle National Biodefense Institute (BNBI) manages and operates the National Biodefense Analysis and Countermeasures Center (NBACC) for the DHS. NBACC was established to address gaps in knowledge about biological agents that could cause harm to citizens and develop and apply forensic protocols to identify the means, method, and forensic signatures associated with a biological agent from a biocrime or bioterrorist investigation (http://www.bnbi.org/). It also invests in scientific programs that are crucial to national defense against bioterrorism. Within NBACC, the National Bioforensic Analysis Center (NBFAC) analyzes evidence associated with a biocrime or bioterrorist act to determine the source, origin, and methods of the attack to attain data for attribution. NBFAC is the lead federal facility as designated by Homeland Security Presidential Directive #10, to facilitate technical forensic analysis and interpretation of data from a biocrime or bioterrorist event (http://www.bnbi.org/).

In 2006, the National Institute for Microbial Forensics & Food and Agricultural Biosecurity (NIMFFAB) was established by Dr. Jacqueline Fletcher at Oklahoma State University (http://entoplp.okstate.edu/nimffab/home). This is the first program devoted to assessing and improving national capabilities in microbial forensics as it relates to plant pathogens and food safety. Other goals of the Institute include establishing working relationships with federal and state agencies to encourage funding programs for technology development related to microbial forensics and agricultural biosecurity, and developing training and educational opportunities related to agricultural biosecurity for students and stakeholders. Finally, it is a goal of NIMFFAB to play an integral part in collaboration, cooperation, communication, and outreach efforts related to microbial

forensics and agricultural biosecurity/food safety (http://entoplp.okstate.edu/nimffab/about.htm).

In 2008, NIMFFAB became a spoke lab for NBFAC, establishing a framework for plant bioforensic capability within the NBFAC laboratory. The initial objective was to adapt, and then test and validate real-time PCR protocols, developed for human pathogens at NBFAC, to high-threat plant pathogens (James et al. 2013). NBFAC's standard operating procedures (SOPs) for sensitivity (limit of detection), specificity, range/linearity, and false positive/negative rates were followed. This working relationship between a University and a federal agency facilitates research and development, and contributes to the improvement of our nation's microbial forensic programs related to food and agricultural biosecurity.

Other research areas within NIMFFAB include food safety, bioinformatics, vector entomology, and diagnostics. In keeping with the land grant mission at Oklahoma State University, NIMFFAB participates in outreach activities to educate the public about food safety and agricultural biosecurity. For the last few summers in Oklahoma, NIMFFAB, along with the USDA ARS, hosted a summer plant pathogen forensics workshop for 4H youth in which they investigated a mock agricultural crime scene and learned to use forensic procedures such as collecting evidence, interviewing suspects, processing the evidence at the lab, and performing lab experiments. They also presented their evidence in a court room proceeding.

Microbial forensic technologies adapted to plant pathogens

Plant pathogen forensics combines microbial forensics and plant pathology to create a discipline that enhances capabilities in agricultural biosecurity in the United States. Technologies such as PCR, DNA sequencing, and mass spectrometry that are used for forensics are also used in 'traditional' plant pathology. The difference however, is the rigorous validation of an experiment necessary for a forensic investigation. With the cooperation of government agencies such as the FBI and DHS, bioforensic assays developed and validated for human pathogens can be adapted for plant pathogens. In 2008, NIMFFAB became a spoke lab for NBFAC and was contracted to test and validate bioforensic assays that were already developed for human pathogens such as *Bacillus anthracis* and *Francisella tularensis*. The goal of the project was to develop real-time PCR bioforensic assays for plant pathogens considered high importance (James et al. 2014). *Xylella fastidiosa* was chosen as a model for plant pathogenic bacteria because of the impact it has on the grape and citrus industries in the U.S. and other parts of the world.

III. *Xylella fastidiosa*

Biology

Xylella fastidiosa is a Gram-negative, xylem-limited, and fastidious bacterium that causes leaf scorch diseases in many plants and can cause major economic losses in grapevines, citrus, and trees such as almond, plum, pear and oak (Chatterjee, 2008). Infection by *X. fastidiosa* is tissue specific and its location in the plant influences symptomatology (Purcell and Hopkins, 1996). For example, in many leaf scorch

diseases bacteria aggregate in leaf veins and petioles (Hearon et al. 1980). Die-back is a common symptom in trees and bacteria accumulate in the branches or the trunk (McGovern and Hopkins 1994). In susceptible grapevines, *X. fastidiosa* multiplies and spreads from the point of infection, moving through the xylem by way of sap flow. There, it attaches to the vessel walls and aggregates to form occlusions within the xylem, blocking water flow (Chatterjee 2008).

Xylem sap has very low concentrations of the organic compounds that most organisms need to survive; however, amino acids and other organic and inorganic substrates are available (Purcell and Hopkins, 1996). Certain amino acids, such as glutamine, asparagine, and cysteine, have been added to media to promote growth of *X. fastidiosa* (Almeida and Purcell, 2003). Xylem sap concentration differs with plant age, growing season, time of day, plant stresses, and fertilization (Andersen and Brodbeck, 1991). These features could explain why *X. fastidiosa* thrives, especially if the plant is under stress and therefore is vulnerable to the advancement of the bacterium.

Taxonomy

X. fastidiosa (Wells et al. 1987) belongs to the family Xanthomonadaceae. The taxonomy of *X. fastidiosa* has evolved over time. In 1973, a bacterium associated with Pierce's disease (PD) of grapevines was described as "rickettsia-like" because of its morphological similarities to members of the Rickettsiaceae (Hopkins and Mollenha, 1973). The "PD bacterium" was first isolated on a medium containing hemin chloride and bovine serum albumin that was supposedly specific for *Rochalimaea quintana*, a rickettsia that causes trench fever (Davis et al. 1978). Despite apparent similarities

between these two bacteria, DNA studies showing that the G+C content of the "PD bacterium" was higher than that of *R. quintana*, which suggested there were differences at the DNA level (Wells et al. 1987). As a result, researchers began referring to these bacteria as fastidious, gram-negative, xylem-limited bacteria (XLB).

Twenty five strains of XLBs isolated from ten plants, including infected grapevine and several tree species having leaf scorch symptoms, were compared using molecular and biochemical techniques (Wells et al. 1987). Fatty acid profiles showed saturated and odd-numbered carbon straight chains to be 18.2% higher in the XLB than in other Gram-negative genera tested, including *Pseudomonas syringae*, *Xanthomonas campestris* and *Erwinia amylovora*, indicating that the XLB comprise a homogenous group of related taxa. Furthermore, in DNA hybridization experiments the PD strain PCE-RR was 99% similar to the plum leaf scald strain and 85% similar to the periwinkle strain, indicating that these XLBs are a single species (Wells et al. 1987). The 16S rRNA sequences of the XLBs contained nucleic acid signatures demonstrating that xanthomonads are the closest known relatives, and excluding any relatedness to the rickettsiae. Based on this information, Wells et al. (1987) proposed the name *X. fastidiosa* for the xylem-limited bacteria.

Twenty six strains of *X. fastidiosa* were classified into three subspecies based on DNA-DNA relatedness of the 16S-23S ITS region (Schaad et al. 2004). Group A consisted of strains from grape, almond, alfalfa, maple, and almond; group B of strains from peach, plum, and sycamore; and group C of only citrus strains. To distinguish an organism as a new species or subspecies, phenotypical and/or serological characteristics must confirm molecular studies (Brenner et al. 1982). The 26 *X. fastidiosa* strains were

grown on several substrates and colony growth characteristics were compared. Strains of taxon group A grew faster on Pierce's disease agar (PD medium) and buffered charcoal yeast extract (BYCE) than did those in groups B and C, which grew faster in periwinkle medium (PW). Strains in groups B and C were susceptible to penicillin, where as those in group A were resistant (83). Based on these characteristics, group A was designated *X. fastidiosa* subsp. *piercei*; B was named *X. fastidiosa* subsp. *multiplex*; and C became *X. fastidiosa* subsp. *pauca*.

Pathogenesis

Early studies to determine how disease is caused by the bacterium in the plant were not conclusive and there is still debate on whether symptoms caused by *X. fastidiosa* are due to water stress resulting from a plant activating tyloses, or if the bacterium is producing a phytotoxin that leads to the scorching symptoms (Goodwin et al. 1988, Perez-Donosis et al. 2007, and Daugherty et al. 2010), although phytotoxins from pure cultures of *X. fastidiosa* may cause symptoms associated with leaf scorch diseases (Lee et al. 1982). When phytotoxin activity from *X. fastidiosa* isolates from infected grapevines was bioassayed by exposing detached leaves to fractions of the phytotoxin (Lee et al. 1982), susceptible grape and tolerant grape cultivars showed leaf scorch symptoms 6-12 hours and 48-72 hours post-inoculation, respectively. Two fractions of phytotoxins recovered by chromatography had different characteristics; fraction 1 produced primarily wilting symptoms without necrosis while fraction 2 consistently produced typical scorching symptoms and necrosis around the leaf margin, but no wilting symptoms (Lee et al. 1982). There is evidence to suggest that the compound ethylene, triggers vascular occlusions in plants when they are infected with *X.*

fastidiosa (Perez-Donosis et al. 2007). For instance, grapevines that were infected with *X. fastidiosa* produced higher levels of ethylene in the leaves compared to the level of ethylene production produced by grapevines that were healthy (Perez-Donosis et al. 2007).

Goodwin et al. (1988) examined whether impacts on water flow through the xylem after *X. fastidiosa* infection was the main factor in symptom development. Water flow rate of *X. fastidiosa* in the xylem was 266 times greater in the healthy control plants than in necrotic plant tissues from infected Chardonnay grapevines. In the latter, water flow was sometimes undetectable, suggesting that *X. fastidiosa* induces water stress on the plant. They also examined the role of phytotoxins in disease symptoms of grapevines. Marginal leaf necrosis occurred after inoculation of healthy grape cuttings with crude phytotoxins from *X. fastidiosa*. Thus, phytotoxins may play a role in disease progression (Goodwin et al. 1988).

X. fastidiosa resides in the xylem of plants but the mechanism of entry into the xylem vessels remains unclear. Reddy et al. (Reddy et al. 2007) suggested that *X. fastidiosa* produces an array of polysaccharide-degrading enzymes including polygalacturonase (PG) to digest the pit membranes of the xylem. The complete genome sequence of *X. fastidiosa* (Van Sluys et al. 2003) revealed a single copy gene (PD1485) that encodes a PG. When pathogenicity assays were performed on grapevines infected with the PD strain "Fetzer", in which the *pglA* gene encoding a PG was knocked out (Reddy et al. 2007), bacterial movement in petioles was restricted for $pgAl^-$ mutants compared to that of the wild-type *pglA* strains. At 14 weeks post-inoculation the bacterium was detected 25 cm from the point of inoculation in 100% of the inoculated

plants compared to 30% of the plants inoculated with the mutant (Reddy et al. 2007). This result seems to indicate that PGs are critical to *X. fastidiosa's* ability to colonize xylem tissue.

Moving waste, toxins, and virulence factors against a concentration gradient requires energy produced by the bacterium (Sharff et al. 2001). In *Escherichia coli* the TolC protein functions as an export mechanism to help the bacterium eliminate harmful toxins (Nikaido, 1996). Many homologs of TolC are present among a wide range of gram-negative bacteria, including *X. fastidiosa* (Sharff et al. 2001). The genome sequences of both the CVC and PD strains of *X. fastidiosa* CVC and PD (Van Sluys et al. 2003) contain genes for multiple hemolysins and type I secretion systems as well as a single TolC family homolog (Reddy et al. 2007). *X. fastidiosa* requires *tolC* for pathogenicity; when the gene is inactivated, infected grapevines show no PD symptoms (Reddy et al. 2007). However *tolC*$^{-}$ mutants could not be recovered after inoculation into grape xylem, indicating that *tolC* is required for pathogen survival.

Relationship of X. fastidiosa with its insect vector

Three essential steps are required for transmission of *X. fastidiosa* into the plant by the insect; 1) the bacterium must be acquired from an infected plant, 2) the bacterium must attach itself to the cuticle of the foregut and colonize that surface and 3) the insect must then transmit to susceptible host (Chatterjee et al. 2008). The flow of sap from the plant to the feeding insect is rapid; sharpshooters can ingest over 100 times their body weight (Mittler, 1967). The *X. fastidiosa* possess Type I pili, which may play a role in attachment to the insect gut (De La Fuente et al. 2007). *X. fastidiosa* colonized the

foregut of the sharpshooters after one day and four day acquisition access periods on infected plants (Almeida, 2005). Feeding behavior is not well understood but there is indirect evidence that transmission of the bacterium into the xylem of the plant occurs at least in part during probing events (Almeida, 2005).

The biology of *X. fastidiosa* is not completely known, but DNA sequencing may give more insight. Whole genome sequences from four strains of *X. fastidiosa* (Xf), 9a5c (citrus), Ann 1 (oleander), Dixon (almond), and Temecula 1 (grapevine), were compared to reveal similarities and differences between them to assess the genetic diversity and strain divergence (Doddapaneni et al. 2006). Among the four strains, 9a5c had the greatest number of strain specific genes (241 genes) followed by Ann 1 (145 genes), Dixon (96 genes), and Temecula 1 (10 genes). Because strain Temecula 1 has the fewest strain specific genes, it could be the ancestral strain of *X. fastidiosa*. With the most strain specific genes, (Xf) 9a5c could be evolving at a faster rate compared to the other strains (Doddapaneni et al. 2006).

Host range and geographical distribution

X. fastidiosa has a vast host range that includes 28 families of monocotyledonous and dicotyledonous plants (Hopkins, 1989). Not all hosts show disease symptoms and among the natural hosts that harbor *X. fastidiosa* are weeds, grasses, and trees (Raju et al. 1983);(Hopkins and Adlerz, 1988). *X. fastidiosa* subsp. *piercei*, causing Pierce's disease (PD), is present in almost all grape growing areas in the United States (Hopkins 1989). *X. fastidiosa* subsp. *multiplex*, which causes bacterial leaf scorch (BLS) on trees, is not restricted to moderate climates as much as PD strains are. BLS has been reported on

elm, oak, sycamore, red mulberry and maple in the northeast and southeast United States (Sherald and Kostka, 1992). *X. fastidiosa* subsp. *pauca* causes citrus variegated chlorosis, which was first reported in Brazil (Paradela et al. 1997).

Development of diagnostic assays for X. fastidiosa

Polymerase chain reaction (PCR) was implemented to detect different strains of *X. fastidiosa* from different hosts. *X. fastidiosa* (PD, multiplex , and CVC strains) were detected by PCR with primer set Rst 31 and Rst 33, with a sensitivity level 100-fold greater than by ELISA (Minsavage et al. 1994). The limit of detection with ELISA was 2 x 10^4 to 1 x 10^5 cfu/ml, while that of PCR was at 2 x 10^2 to 1 x 10^3 cfu/ml. Furthermore, a positive ELISA test required 4,000 cfus of *X. fastidiosa*, while PCR required only 3-4 cfus. The primers reported by Minsavage et al. (1994) were some of the earliest primers for detecting *X. fastidiosa* and since then, several other primers have been designed and are able to detect as little as 1-10 fg of DNA (James et al. 2014, Ouyang et al. 2013).

A multiplex PCR assay was developed to detect *X. fastidiosa* DNA from a DNA mixture of multiple species infecting grape, almonds, and oleander (Hernandez-Martinez, 2006). When primer sets; XF2542-L/R (designed to amplify PD strains tested), XF1968-L/R (designed to amplify only oleander strains), and ALM1/2 (designed to amplify multiplex strains) were used for this assay, one 412-bp band was observed. Using DNA extracted from infected oleander tissue a 638-bp band was observed; while mixture of grape and oleander DNA used as the template yielded two bands, corresponding to the grape strain and oleander strain, 412-bp and 638-bp, respectively. When DNA from infected almond was used in the multiplex PCR reaction, some samples yielded a 412-bp band while others yielded three bands of 638-bp, 521bp, and 412-bp.

These results suggested that there may be two genotypes of *X. fastidiosa* strains that cause almond leaf scorch (Hernandez-Martinez, 2006).

X. fastidiosa strains can be detected and differentiated using melt curve analyses with SYBR® green real-time PCR technology (Bextine and Child, 2007). PCR primers were designed using the sequence of the *gyrase B* (*gyrB*) gene, which is conserved among strains of *X. fastidiosa* but diverse enough to discriminate among similar strains (Yamato and Harayama, 1995). Using SYBR® Green and melting temperature melting temperature (Tm) profiles, all eight *X. fastidiosa* PD strains were so identified, and all six ALS and OLS strains were identified as such by T_m melt curves. The T_m difference between PD and OLS strains was 0.3°C, T_m between OLS and ALS strains was also 0.3°C, but PD and ALS strains were separated by 0.6°C (Bextine and Child, 2007).

Impact of X. fastidiosa CVC on the United States

Recently, *X. fastidiosa* subsp. *pauca*, which causes CVC on citrus plants, was used to analyze agricultural biosecurity in the U.S. (Ancona et al. 2010). This species is a select agent, it is highly regulated, and it can be a good model for other high risk plant pathogens (Fletcher et al. 2006). Further, *X. fastidiosa* strains already are causing economic damage to vineyards, and other domestic crops (Hopkins, 1989). *X. fastidiosa* subsp. *pauca* is non-native but, if introduced into the U.S. could cause devastating economic loss among the citrus industry. The disease affects the leaves, which become chlorotic, and the fruits, which remain small, ripen prematurely, and have hard rinds (Brlansky et al. 1991). Even though the trees rarely die, productivity is minimal.

Oranges are highly valued for both production in, and exportation from, the U.S. Currently the entire citrus industry in the U.S. is threatened by the disease huanglongbin

(HB), also known as citrus greening (http://www.ars.usda.gov/citrusgreening/). HB was first detected in Florida in 2005, and orange production in the U.S. dropped dramatically from 2004. Since then, orange production has been inconsistent (USDA Citrus Fruits 2013 Summary). During the 2012-2013 season, orange production decreased 21 percent from the previous year, but in 2008, its production was up 32 percent from the year 2006-2007. If the CVC strain of *X. fastidiosa* were to be established in the United States the citrus industry will be even more threatened.

PCR detection methods for X. fastidiosa CVC strains

Because the CVC strain of *X. fastidiosa* is destructive to the citrus industry in the United States and several other citrus growing countries around the world, robust and reliable detection methods for it are essential. Polymerase chain reaction (PCR) is often a standard for simple pathogen detection because it is cost effective, reproducible among labs, and rapid. PCR methods have been established for not only discriminating *X. fastidiosa* CVC strains from other *Xylella fastidiosa* strains, for example Pierce's disease (PD) strains, but for discriminating among CVC strains.

Oliveira et al. (2002) developed a rapid, simple and reproducible quantitative PCR (qPCR) assay to detect *X. fastidiosa* strains isolated from citrus. RT-PCR primers and a probe developed from the genome of the 9a5c CVC strain (Van Sluys et al. 2003) were specific for nine *X. fastidiosa* isolates from infected sweet orange trees and all nine were amplified. In contrast, *X. fastidiosa* DNA isolated from grape, periwinkle, plum and coffee were not amplified (Oliviera et al. 2002).

A recent study by Li et al. (2013) compared new and previously published primers and probes for detecting *X. fastidiosa* species including the ones causing citrus

variegated chlorosis (CVC) with a their own set of primers and probes. The purpose of the study was to develop and validate a qPCR assay that is standardized and specific for CVC strain discrimination because, over the last 20 years in which PCR protocols have been developed for detecting *X. fastidiosa* species, researchers used different protocols and reagents. These new primers and probes detected all 36 *X. fastidiosa* strains with a limit of detection was equivalent to 2-10 cells of *X. fastidiosa* per reaction. Furthermore, the primers and probes specific for the CVC strains only amplified those strains (Li et al. 2013). As previously mentioned above, CVC is a destructive disease and reliable and robust detection assays that are standardized are needed especially in the case of microbial forensics.

Immunological-based assays for detection of X. fastidiosa CVC

CVC strains are also detected with immunomolecular assays such as immunocapture-PCR (IC-PCR) and immuno-PCR (I-PCR). I-PCR differs from IC-PCR in that bacterial cells are not captured by specific antibodies, but specific antibodies are conjugated with nucleic acid, and then PCR is performed. These assays were more sensitive and less labor intensive than either ELISA or conventional PCR due to the fact that nucleic acid extraction of plant material is unnecessary (Peroni et al. 2008). IC-PCR had a limit of detection of 10^3 cells, 10-fold lower than that of ELISA. Detection limits from Immuno-PCR assays were 10^1 bacterial cells, 100-fold lower than that for IC-PCR and 1000-fold lower than that for ELISA (Peroni et al. 2008).

IV. *Fusarium proliferatum* overview and use as a model fungal plant pathogen for investigating microbial forensic issues

Taxonomy

Fusarium proliferatum (Matushima) Nirenberg 1976, in the phylum Ascomycota, was first described as *Cephalosporium proliferatum* (Matsushima, 1971), but later was reclassified as a unique species (Nirenberg, 1976). Prior to 1976, many of the *F. proliferatum* isolates were identified as *F. moniliforme* (Leslie and Summerell, 2006). As more information about host range and morphological characteristics were determined, *F. moniliforme* was resolved into *F. proliferatum, F. anthophilum, F. subglutinans, F. circinatum, F. sacchari, F. verticillioides, and F. guttiforme* (Leslie and Summerell, 2006, Nelson et al. 1983, Nirenberg and O'Donnell 1998). The teleomorph (sexual state) of *F. proliferatum* was identified as *Gibberella fujikuori* var. *intermedium* (Kuhlman, 1982) and later renamed *G. fujikuori* mating population D, based upon electrophoretic karyotype differences, synthesis of secondary metabolites, sensitivity to antifungal agents or the ability to form a heterokaryon (a form having multiple nuclei per fungal cell) (Leslie, 1995).

Host range

Fusarium proliferatum'*s* host range, the widest of all described species of *Fusarium*, includes onion, mango, wheat, maize, asparagus, palm, pine, and rice (Proctor et al. 2010). The fungus has been isolated from about 75 plant species, including monocots, dicots, and conifers; however, *F. proliferatum* causes disease in only half of them (Proctor et al. 2010). The fungus can also be isolated routinely from grass species

Andropogon gerardii, A. scoparius, and *Sorghastrum nuttans* in North American tallgrass prairies (Leslie et al. 2004). Although plant pathogens generally do not cause disease in humans, there is a reported case of *F. proliferatum* causing the death of an immunocompromised human patient (Summerbell et al. 1988). *F. proliferatum* is resistant to most antifungal drugs, including amphotericin B and posaconazole (Herbrecht et al. 2004, Pujol et al. 1997).

Geographic distribution

Fusarium proliferatum occurs worldwide and has been reported in the northwest, central and eastern parts of the United States (Leslie et al. 1990, Palmero et al. 2012), the Middle East (Alizadeh et al 2010, Bayraktar and Dolar, 2011, Iqbal et al. 2006); Europe (Gherbawy et al. 2001);(Logrieco et al. 1995); (Stankovic et al. 2007); (Palmero et al. 2010), South America (Sampietro et al. 2010), and Japan (Dissanayake et al. 2009).

Biology

Fusarium proliferatum is a soilborne fungus. The morphological characteristics of closely related *Fusarium* species are very similar and molecular diagnostic tools are often required for species discrimination. *F. proliferatum* can be distinguished from *F. oxysporum* by its production of chains of microconidia (Leslie and Summerell, 2006), and by the absence of chlamydospores, overwintering structures common to most other species(54). *F. proliferatum* also produces polyphialides, in which chains of microconidia arise, a feature absent in *F. verticillioides* and *F. thapsinum*, which, like *F. proliferatum*, were resolved from *F. moniliforme* (Leslie and Summerell, 2006). The sexual stage (teleomorph) of *F. proliferatum*, *Gibberella fujikuori* var. *intermedia* (Kuhlman, 1982), is

one of over 40 phylogenetically distinct lineages that comprise the *Gibberella fujikuori* species complex (O'Donnell et al. 2000). *G. fujikuori* var. *intermedia* is closely related to *G. fujikuori* var. *moniliformis* and *G. fujikuori* var. *subglutinans*, but it can be distinguished from the latter by its smaller ascospores (O'Donnell et al. 2000). *Fusarium* species are differentiated also by mating-type tests under the appropriate conditions (Leslie and Summerell, 2006).

Mycotoxins produced by F. proliferatum

Two main categories of mycotoxins produced by *Fusarium* are fumonisins and trichothecenes. Notable trichothecenes, such as deoxynivalenol (DON), nivalenol, and T2 toxin (Bluhm et. al., 2002) have toxic effects on animals that include growth retardation, reduced ovarian function, immunosuppression , feed refusal, and vomiting (Rocha et al. 2005). Fumonisins are cytotoxic and carcinogenic to animals and humans. Although physiological effects are not fully understood, there is evidence to suggest that they interfere with metabolic functions and disrupt the urea cycle (Hopkins and Adlerz, 1988). Like other *Fusarium* species, *F. proliferatum* produces several other mycotoxins, including beauvericin, and moniliformin, first recovered from maize in Italy (Logrieco et al. 1995), and small amounts of gibberellic acid (Tsavkelova et al. 2008). Although mycotoxin contamination in maize receives a great deal of attention, other crops vulnerable to contamination include asparagus, onion, and garlic (Waskiewicz et al. 2009); (Stankovic et al. 2007). *F. proliferatum* also colonizes many prairie grasses, where mycotoxins may impact grazers such as bison, elk, and others (Leslie et al. 2004).

Disease cycle

Diseases caused by *F. proliferatum* include rots, diebacks, blights, and wilts (Proctor et al. 2010). Rots, which can occur on roots, bulbs, crowns, stems, shoots, fruits and seeds, receive the most attention. *F. proliferatum* can infect some hosts without causing disease symptoms, a phenomenon reported on maize, orchids and wheat (Jeney et al. 2007, Kwon et al. 2001, Tsavkelova et al 2008). Examples of rots caused by *F. proliferatum* are stalk and ear rot of corn and root and stem rots of nongrain crops. The fungus can overwinter as perithecia or mycelium, usually in corn stalk debris, and germinate when environmental conditions are favorable. In the spring, warm and wet conditions allow for the dispersal of ascospores, which are carried by the wind to corn stalks or ears (Agrios, 1997). Conidia can form on infected plant parts and serve as a source of secondary inoculum and spread by wind to nearby plants or fields where the infection process starts again. At the end of the host's growing season the fungus can overwinter on dead stalk debris (Agrios, 1997) for up to 630 days on the soil surface or at depths of 15 to 30 feet below the soil (Cotton and Munkvold, 1998).

F. proliferatum, **the causal agent of salmon blotch of onion in Israel, as a model for validation of plant pathogen forensic analyses.**

Fusarium proliferatum on onions in Israel

In the summer of 2005-06, pink discoloration was observed on the surface of some white onions in commercial fields located in Yotvata. When Daryl Gillette, head vegetables researcher at the Southern Arava Research and Development in southern Israel, peeled away the outer layer, discoloration continued on the inner 2^{nd}, 3^{rd}, 4^{th}, 5^{th},

and 6th layers. The onions eventually rotted. Whether these symptoms represented a primary or secondary infection was unclear. A fungus was consistently isolated from symptomatic bulbs and re-infected onions, causing identical symptoms, fulfilling Koch's Postulates. The fungus was identified by PCR as *Fusarium proliferatum* (Gamliel, personal communication).

Creating a decision tool to determine if an outbreak of *F. proliferatum* is naturally occurring or due to human involvement

One question that must be answered before a forensics investigation is "has a crime been committed?" (Rogers, 2011). Answering this question in an agricultural setting from a plant pathology perspective can be complicated since most growers and plant pathologists do not associate plant diseases with intentional acts. Another factor that makes answering this question difficult is that plant disease symptoms do not show up immediately upon infection, but can take several weeks. When growers notice disease in their fields, they may or may not be quick to employ containment and mitigation strategies (Fletcher et al. 2006).

A tool designed to assist investigators in determining whether a disease outbreak was due to natural events or to human involvement could shorten the time for a response to a biocrime. Such a decision tool was developed to confirm or rule out the use of biological warfare in the case of an unusual epidemic of tularemia in Kosovo from 1999-2000 (Grunow and Finke, 2002). A set of criteria was described and a numerical value was assigned to each. In that case, application of the tool ruled out the possibility that the

epidemic had resulted from an intentional release of the bacterium *Francisella tularensis* (Grunow and Finke, 2002).

A decision tool suitable for the investigation of an outbreak of a plant disease was developed based on similar principles (Rogers 2011). Criteria included factors relevant to the pathogen host range, environmental conditions, epidemiology, dissemination, and other disease-relevant elements. This tool was designed and validated using a specific plant disease model, wheat streak mosaic, caused by *Wheat streak mosaic virus* (WSMV) (Rogers 2011). It would be useful to develop additional tools based on other plant diseases, particularly those having significantly different features and pathogens, to extend the concept of the decision tool. Ultimately, it might be possible to construct a generic plant disease tool that could be used in a variety of scenarios (Rogers 2011). The fungus *Fusarium proliferatum* is a good candidate for this application for several reasons. It has a very broad host range (Proctor et al. 2010), and it, along with closely related *Fusarium* species, produce mycotoxins, such as fumonisins and trichothecenes, that can be harmful to animals and immunocompromised humans (Abbas et al. 1998); (Hussein and Brasel, 2001).

Fusarium proliferatum **detection and strain differentiation as a model system to validate technologies developed for plant pathogen forensics**

The goal of a forensics investigation is the attribution of a crime to the perpetrators. Generally, when a crime involves a pathogen or other microbial agent, investigators will seek to match microbial strains found at the crime scene to strains associated with a suspect.

Using *F. proliferatum* as a model for plant pathogen forensics will require the ability to accurately identify and discriminate among fungal isolates collected in a variety of locations. Unique or location-specific genetic signatures found in fungal populations collected at a crime scene or other relevant location can help lead investigators to the point of origin of that isolate. Morphological characteristics of *F. proliferatum* can be used to identify the fungus based on the presence of small chains of microconidia formed by polyphialides; however, several other species of *Fusarium* have similar morphology. Several methods have been employed for detection of *F. proliferatum* primarily PCR for quick and rapid screening of contaminated grains or crops.

Polymerase chain reaction (PCR) as a tool for detecting Fusarium proliferatum

PCR, using primer sets for amplification of histone and β-tubulin gene sequences from *Neurospora crassa* (Glass and Donaldson, 1995), was used as a detection and strain differentiation tool to characterize multiple *Fusarium* species isolated from conifers (Donaldson et al. 1995). A "housekeeping" gene region, the ribosomal internal transcribed spacer (ITS) region, was used as a control because it is conserved among all Ascomycetes but different enough to separate fungi at the genus level and also considered the fungal barcode (White et al. 1990). *Fusarium* species-specific primers would be ideal for quick, high-throughput screening during a forensic investigation, in part because a crop could harbor multiple pathogens including other *Fusarium* species. A primer designed from a single copy gene, calmodulin, (Mule et al. 2004) distinguished among *F. proliferatum*, *F. subglutinans*, and *F. verticillioides* with a limit of detection of 12.5 pg of DNA per PCR reaction. Greater PCR sensitivity was achieved by using the multi-copy IGS (intergenic spacer of rDNA) gene (Jurado et al. 2006). Since mycotoxins

produced by *Fusarium* species pose a risk for animal and human health, PCR screening for specific mycotoxin-producing species is necessary, and primers developed to amplify *Fusarium* toxin biosynthetic genes are species-specific (Sampietro et al. 2010). For example, *F. proliferatum* can be detected with primers targeting the FUM1 gene, but not with primers designed to target a trichothecene gene because the fungus does not produce the latter.

Quantitative real-time PCR (qPCR) assays are used for the detection and discrimination of multiple species of *Fusarium* that are frequent contaminants of cereal grains (Bluhm et al. 2004), (Bluhm et al. 2002), (Nicolaisen et al. 2009). This detection method allows for faster run times than end-point PCR due to shorter product sizes, increased target specificity and sensitivity, and gel electrophoresis often is not necessary (Bluhm et al. 2004). The translation elongation factor 1-α (TEF-α) gene, the marker of choice for molecular identification of *Fusarium* (Geiser et al. 2004), has been used in real-time PCR assays. Using this gene, eleven *Fusarium* species, including *F. proliferatum,* could be detected from wheat and maize field samples (Nicolasisen et al. 2009). Multiplex real-time PCR, which can be an ideal method for quick, reliable, and high throughput screening for mycotoxins in cereal grains is very similar to qRT-PCR, but uses two or more primer sets to amplify a mixed DNA template. Bluhm et al. (2004) demonstrated that seven species of *Fusarium* could be detected and distinguished from contaminated grains using the mycotoxin biosynthetic gene primers, TRI6 and FUM1.

DNA fingerprinting methods to discriminate F. proliferatum from other Fusarium species and to discriminate among isolates of F. proliferatum.

Gene regions other than ITS have been explored for differentiating strains of *F. proliferatum*. Mitochondrial small subunit rDNA (mtSSU rDNA) sequences have been analyzed for many *F. proliferatum* isolates, and although mtDNA evolves at a rapid rate compared to other gene regions it is stable among populations for several generations (O'Donnell et al. 1998); (Laday et al. 2004). Distinct mtDNA-RFLP fingerprints were obtained from isolates from maize, asparagus, palms, and reed and each banding pattern can be categorized into a mating type (Laday et al. 2004). PCR has limited ability to differentiate isolates of the same species, so more discriminatory molecular methods are needed. Restriction fragment length polymorphism (RPLP) creates a genetic "fingerprint" of an individual by digesting its DNA with restriction enzymes and observing gel electrophoresis band patterns or hybridizing blotted digested DNA with a specific probe (Cooke, 2005). When the ITS region of DNA from *Fusarium* isolates obtained from nursery-grown conifers were targeted for RFLP analysis, four of six fungal species were differentiated (Donaldson et al. 1995). The ITS region also can be used as a taxonomic discriminator between *F. verticillioides* and *F. proliferatum.* ITS-RFLP profiles revealed that all *F. verticillioides* and all *F. proliferatum* isolates fell into two groups designated A and B, respectively. The ITS amplicon sequences between isolates from groups A and B differed by a 6 bp insertion within the ITS gene region (Visentin et al. 2009).

Random amplified polymorphic DNA (RAPD), a PCR-based fingerprinting technique, amplifies random DNA sequences throughout a genome (Cooke, 2005). To obtain meaningful strain discrimination this technique requires numerous primers and high variation in the data sets (Soll, 2000). RAPD analysis was performed to determine

genetic variability among isolates of *F. mangiferae* from mango grown in Pakistan (Iqbal et al. 2006), 45 random decamer primers amplified, on average, 7.86 bands per primer set, ranging from 250 bp to 3,000 bp in size. They found that there were genetic differences among the populations of *F. mangiferae* collected from different regions within Pakistan, but also they had strains from different regions grouping together indicating the possibility that there is pathogen movement.

Variable number tandem repeats (VNTRs), short repetitive nucleotide sequences, have different copy numbers in different bacteria (Cooke, 2005). Typing multiple VNTRs at the same time (multiple-locus VNTR analysis (MLVA), yields a fingerprint (Keim, et al. 2000). A program designed to locate tandem repeats can be used to search the entire fungal genome sequences (Benson, 1999). The full genome of *F. graminearium* has been sequenced (http://www.broad.mit.edu) and, VNTR markers, designed for *F. graminearum* and *F. asiaticum* (Suga et al. 2004) were chosen based on distinct polymorphisms from 54 loci in *Fusarium* strains from the United States, Italy, and China. It is possible to develop such markers for other *Fusarium* species like *F. proliferatum*.

Multilocus sequence typing (MLST) compares microorganisms based on a set of genes, usually encoding housekeeping functions rather than focusing on a single gene (Breeze et al. 2005). MLST techniques are reproducible among laboratories but limitations occur when the organisms being evaluated show very little genetic variation. Although few studies on the use of MLST for fungi have been published the technique was used to differentiate the *F. solani* species complex (Debourgogne et al. 2010). *F. solani*, a well-known plant pathogen, garners much attention in the medical field because it is an opportunistic pathogen in humans (Chang et al. 2006). The MLST strategy

involved 25 genes tested in different combinations to yield a 5-locus MLST scheme able to type individuals of *F. solani* (Cooke, 2005). Differentiating among *F. proliferatum* strains in this way may be possible; however, if isolates found worldwide are very similar genetically then using housekeeping genes like ITS, β-tubulin, and TEF1-α may not be effective. However, if unique regions within the species' mtDNA for example, then MLST could provide more insight to the genetic variability of this fungus.

Repetitive genome segments called simple sequence repeats (SSRs), consisting of 2-6 bp repeats occurring in tandem, were used to asses genetic diversity of *Fusarium* species pathogenic to onions in Turkey (Bayraktar et al. 2011). A total of 322 isolates belonging to seven species of *Fusarium,* including *F. proliferatum*, were collected from 223 onion fields. The ISSR (inter-specific simple sequence repeats) analysis of a subset of 70 isolates representing the seven *Fusarium* species, showed distinct banding patterns among the isolates belonging to different species (Bayraktar et al. 2011). When Neumann et al. (2011) examined a population of *F. proliferatum* from root zone soil of *Livistona mariae* palms (planted 20 m apart) from Finke Gorge National Park, Northern Territory, Australia (Neumann et al. 2011), their seventy-seven isolates fell into two genetically similar, but separate, clades. The authors speculated that there could have been two separate introductions of *F. proliferatum,* or a single introduction followed by a split over time into two populations (Neumann et al. 2011). Since the isolates were collected from a national park, they may reflect the natural spatial distribution of *F. proliferatum* in that particular environment.

We do not know the spatial and temporal distribution of *F. proliferatum* in Israel, nor whether there are multiple genotypes of the species within a field or are if they are a clonal population.

SSR markers have been identified in other species of *Fusarium*. For those species having fully sequenced genomes, like *F. verticillioides* (*Fv*), hundreds of SSR loci can be distinguished (Leyva-Madrigal et al. 2014). Four-hundred seventy microsatellite markers were identified among eleven chromosomes of *Fv* and used to obtain many more SSR markers for more robust population biology studies of *Fv*, which is the most common soil inhabitant of the *Fusarium* species (Leyva-Madrigal et al. 2014). Out of the 427 microsatellite markers, only eleven primer pairs were validated with 62 strains of *Fv*; all primer pairs were polymorphic. Santana et al. (2009) used a 454 pyro-sequencing approach for identifying microsatellite loci in genomic DNA of *F. circinatum*, a pathogen of pine trees. Sequenced DNA contigs were assembled and 28 SSR primer pairs were designed and tested for polymorphisms with a collection of *F. circinatum* isolates (Santana et al. 2009). Although the number of isolates used in the study was not reported, 13 primer pairs were polymorphic based on the amplicon sizes. SSR markers have also been tested for the *F. oxysporum* (*Fo*) species complex. Nine SSR primers developed from an isolate of *Fo* were tested with 64 *Fo* isolates from soils and plant material collected from different regions in Ethiopia and the Netherlands (Bogale et al. 2005). Among the 64 isolates, 71 alleles were found using the nine SSR primers, which could be sufficient for further *Fo* genetic diversity studies (Bogale et al. 2005).

The usefulness of SSR markers in population biology and genetic diversity studies is already well established in the oomycete research community. SSR markers

have been used for the common greenhouse plant pathogens *Pythium aphanidermatum*, *P. irregular*, and *P. cryptoirregulare*, for which 14, 22, and 23 polymorphic SSR primers, respectively, were identified (Moorman et al. 2002; Lee and Moorman 2008). The SSR markers revealed a total of three discrete populations for the three *Pythium* species, as well as separating out hybrid isolates between *P. irregular* and *P. cryptoirregulare*, which most likely exchanged DNA over time (Lee and Moorman, 2008). SSR markers for *Phytophthora infestans* were used for a one-step multiplex PCR assay (Li et al. 2013) in a cooperative, international effort to standardize SSR multiplex PCR protocols for *P. infestans* worldwide. Instead of visualizing amplicons on a gel, the SSR primers were labeled with a fluorescent tag (Li et al. 2013). Scientists in Great Britain and the Netherlands validated and standardized the multiplex SSR PCR assay with 96 *P. infestans* isolates collected between the years 2001-2011 (Li et al. 2013). They found 80 different fingerprints among the 96 isolates and were able to identify isolates having different ploidy levels in their genomes. *P. infestans* is normally a diploid organism, but in nature recombination can occur between isolates to form hybrids having 3 copies of a DNA (Li et al. 2013).

The fact that SSR markers have been used for some *Fusarium* species and their potential usefulness for genetic and population biology studies, demonstrated in the oomycete research community, made this technology a good choice for characterizing *F. proliferatum* isolates collected from Germany, Austria, North America, and Israel. The DNA fingerprinting techniques described above can be used effectively to not only detect but to differentiate *Fusarium* species from one another, as well as discriminate between isolates of the same species. Most studies on *F. proliferatum,* focus on isolates from a

single geographical region (Bayraktar et al. 2011, Donaldson et al. 1995, Waskiewicz et al. 2009, and Iqbal et al. 2011).

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CHAPTER III

Validation of real-time PCR assays for bioforensic detection of model plant pathogens

The work presented in this chapter has been published as a multi-author, refereed paper in the *Journal of Forensic Sciences*. This chapter preface defines the work done specifically by Mr. Ian Moncrief.

The National Institute for Microbial Forensics & Food and Agricultural Biosecurity at Oklahoma State University was contracted by the U.S. Department of Homeland Security's National Bioforensic Analysis Center (NBFAC) to test and validate bioforensic real-time PCR assays for the plant pathogens *Xylella fastidiosa*, *Pseudomonas syringae* pathovar *tomato*, and *Wheat streak mosaic virus*. If the NBFAC were to investigate a criminal case involving the agriculture sector, then standardized, validated bioforensic assays like the ones presented here, could be employed as a part of the overall investigation (James et al. 2012). *X. fastidiosa* is a Gram-negative, xylem-limited bacterium that causes many leaf scorch diseases in plants and can cause major economic losses, especially in grapevines (Hopkins, 1989). The citrus variegated chlorosis (CVC) strain of *X. fastidiosa* threatens the citrus industry in the United States. Although this strain was once included on the USDA APHIS Select Agent List, during the time of this research, it was being considered for removal. *X. fastidiosa* was chosen

for this bioforensic assay in part because this species occurs in Oklahoma and because the 'CVC' strain places the U.S. citrus industry at risk.

The components of the real-time PCR bioforensic assay developed for *X fastidiosa* include (1) primer design, (2) defining the assay's linearity, range, and limit of reproducible detection, (3) developing an internal positive control, and screening inclusivity and exclusivity panels to establish assay specificity. The isolates of *X. fastidiosa* are listed in Table 1 of the published paper. The PCR primers and a probe specific for *X. fastidiosa* were designed from portions of the 16S-23S ITS region in the *X. fastidiosa* genome. Primer design took into account several factors including amplicon size, GC content, annealing temperature, and the probability of secondary structures forming at the 3' ends of the primers which could inhibit the PCR reactions.

The linear range and sensitivity of the primers were tested by performing ten-fold serial dilutions of the DNA from the 'Temecula' strain of *X. fastidiosa*. Two analysts performed this portion of the assay on different days, as required for the validation. The primers were sensitive in the range of 1 ng to 10 fg for *X. fastidiosa*, and the assays were repeatable and precise as indicated by the CV and the average C_t values (Table 6). A DNA concentration of 10 fg was the limit of detection (LOD) for *X. fastidiosa* (Table 7).

The inclusivity panel consisted of several other *X. fastidiosa* isolates which were tested with the primers designed in this study. All isolates were amplified with the specific primers (Table 2). To make sure that our primers were specific only for *Xylella*, they were tested with an exclusivity panel of DNA from several plants, animals, and near-neighbor microbes. The plants were selected for their economic importance or

placement in diverse taxa (Table 3), the animals for their association with agricultural environments (Table 4) and the near-neighbor microbes for their taxonomic relatedness to *Xylella* or the likelihood of their occurrence in the same environment as *X. fastidiosa* (Table 5). The primers did not amplify DNA from either exclusivity panel, thereby confirming their specificity for *X. fastidiosa*.

The inclusion of an internal positive control in a bioforensic assay adds credibility to the results and strengthening forensic cases. We designed a plasmid that contained (1) the target sequence of *X. fastidiosa* specific for our primers and (2) a restriction enzyme site for *AvaI* which was produced by Integrated Technologies (San Diego, CA). The restriction site allowed for ready discrimination between amplified control and the product produced after restriction enzyme treatment.

Overall, a real-time PCR assay for the detection of *X. fastidiosa* was developed and validated for use in bioforensic investigations. This work, together with the work of other NIMFFAB investigators, demonstrated successful adaptation of forensically valid PCR assays, developed for human pathogens, for use with plant pathogens of high risk to the U.S. agriculture sector.

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Journal article:

Validation of real-time PCR assays for bioforensic detection of model plant pathogens.

ABSTRACT: The U.S. agricultural sector is vulnerable to intentionally introduced microbial threats because of its wide and open distribution and economic importance. To investigate such events, forensically valid assays for plant pathogen detection are needed. In this work, real-time PCR assays were developed for three model plant pathogens: *Pseudomonas syringae* pathovar *tomato*, *Xylella fastidiosa*, and *Wheat streak mosaic virus*. Validation included determination of the linearity and range, limit of detection, sensitivity, specificity, and exclusivity of each assay. Additionally, positive control plasmids, distinguishable from native signature by restriction enzyme digestion, were developed to support forensic application of the assays. Each assay displayed linear amplification of target nucleic acid, detected 100 fg or less of target nucleic acid, and was specific to its target pathogen. Results obtained with these model pathogens provide the framework for development and validation of similar assays for other plant pathogens of high consequence.

The U.S. agricultural system is vulnerable to bioterrorist attack in a variety of food-related sectors including production, processing, and distribution (1,2). Crops are especially vulnerable because of their economic importance, distributed nature, infre-

quent surveillance, extensive monoculturing, heavy dependence on chemical disease control, and the threat of exotic pathogens that have not yet breeched U.S. borders (1).

To prepare for possible biological attacks on U.S. agriculture, a national capability in plant pathogen forensics is needed and should include the adaptation of traditional forensic methods for use with plant pathogens and environmental samples from agricultural settings (3). To this end, the National Institute for Microbial Forensics and Food & Agricultural Biosecurity at Oklahoma State University was contracted by the U.S. Department of Homeland Security's National Bioforensic Analysis Center (NBFAC) to develop and validate real-time PCR assays for bioforensic detection and identification of plant pathogens to aid in attribution in a court of law. Two phytopathogenic bacteria, *Pseudomonas syringae* pathovar *tomato* (P.s. tomato) and *Xylella fastidiosa*, and one plant virus, *Wheat streak mosaic virus* (WSMV) were chosen as convenient models from which the developed technology could be transferred to more threatening pathogens. Furthermore, these pathogens meet certain criteria of potential bioweapons, including toxin production, ease of handling, high infection rate, unavailability of control methods or resistant hosts, lack of reliable detection methods, rate of spread in nature, crop losses associated with disease, and environmental persistence (4). *Pseudomonas syringae*, a common bacterial pathogen, infects a variety of economically important plant hosts including grains, vegetables, fruits, and forest trees, leading to significant economic losses worldwide (5). Infection results in the production of necrotic lesions on aerial portions of the host plant (6). *Pseudomonas syringae* pathovar *tomato*, a variant of *P. syringae* identified based on its host range, infects Brassica species (broccoli, cabbage, etc.) and *Solanaceae* species (tomato, pepper) in which it causes bacterial speck disease

(5,7,8).

Xylella fastidiosa is a fastidious bacterium with a very wide host range that includes 28 plant families (9). The pathogen, which is transmitted by several species of xylem-feeding insects known as sharpshooters, causes leaf scorch diseases and significant losses in economically important hosts such as grapes, almonds, and citrus (10). Strains of *X. fastidiosa* are classified into three subspecies: *X. fastidiosa* subsp. *piercei* causes Pierce's disease of grape, *X. fastidiosa* subsp. *multiplex* causes bacterial leaf scorch of several tree varieties, and *X. fastidiosa* subsp. *pauca*, a former select agent, causes citrus variegated chlorosis (9,11–13).

Wheat streak mosaic virus (WSMV), a member of the family Potyviridae, is found in most wheat-growing regions of the world, where it commonly infects wheat and other grasses leading to significant economic losses (14–16). The pathogen, which is transmitted by the wheat curl mite (*Aceria tosichella*), causes wheat streak mosaic disease (14,17). Symptoms of infection present as fine chlorotic streaks that may turn into severe streaking and mosaic (15).

The objective of this study was to develop real-time PCR assays and assay controls for detection of these model plant pathogens. The assays were then validated for use in microbial forensics investigations by determining their linearity and range, limit of detection, sensitivity, specificity, and exclusivity, which supports third-party peer review and ultimately ISO 17025 accreditation.

Materials and Methods

Nucleic Acid Extraction From Pure Cultures, Plants, and Animal Blood and Tissue

Strains of P.s. tomato (Table 1) were grown in King's B broth at 28°C with shaking at 120 rpm (18). Bacterial DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. DNA from X. fastidiosa was obtained from American Type Culture Collection (Manassas, VA) and from university and governmental laboratories (Table 1).

RNA from WSMV and near-neighbor viruses was extracted from infected plant material using the Qiagen RNeasy Plant Mini Kit and the manufacturer's protocol. First-strand copy DNA (cDNA) was then synthesized from the viral RNA using Molo-ney murine leukemia virus reverse transcriptase (M-MLV RT) and accompanying reagents (Invitrogen, Carlsbad, CA) along with a viral poly A tail primer RCF1 (5′-AGCTGGATCCTTT TTTTTTTTTTTT-3′) according to the manufacturer instructions (19). Copy DNA was purified using the Qiagen QIAquick PCR Purification Kit (Qiagen). Purified cDNA was used in all WSMV assay validation testing.

DNA used in exclusivity testing was extracted from plants and animal blood or tissue using the Qiagen DNeasy Plant Mini and DNeasy Blood & Tissue kits along with their respective protocols.

Primer Selection

Pathogen-specific primers and probe samplified a 100- to 200-bp fragment of the Cor gene in *P.s. tomato,* the 16S-23S ITS region in *X. fastidiosa*, or portions of the P3 and CI genes in WSMV (Table 2). Oligo and probe sequences were designed and analyzed for size, self-complementarity, GC content, and annealing temperature using Primer3 computer software and for the production of secondary structures using the Mfold Web ser-ver (20, 21). Primers and dual-labeled probes were synthesized commercially (Sigma-Aldrich, St. Louis, MO).

Real-time PCR Assays

Amplification reactions were carried out on an ABI 7900HT Real-time PCR system using the ABI TaqMan Gold with Buf-fer A Pack and ABI GeneAmp dNTPs (Applied Biosystems, Carlsbad, CA). Reaction volumes of 50 lL contained 5 lL of template DNA, 5 lL of TaqMan Buffer A, 5 mM $MgCl_2$, 0.3 lM of each primer, 0.25 lM of probe, 0.25 mM of each dNTP, with the exception of dUTP, which was added at a con-centration of 0.5 mM, 3 mg/mL BSA, and 23.83 lL of sterile water. The PCR cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min, with fluorescence measured after each annealing step. Data were analyzed using ABI SDS soft-ware version 2.3 (Applied Biosystems, Carlsbad, CA) with an automatic baseline and a manual cycle threshold (C_t) of 0.2.

Linearity and Range

The linear range and sensitivity of each assay was evaluated by analyzing serial dilutions of DNA extracted from a single strain of each target pathogen. Pathogen strains used included: *P.s. tomato* DC3000, *X. fastidiosa* Temecula, and the type strain of WSMV. Ten-fold serial dilutions of DNA were prepared and tested by two different individuals on different days. Each analyst prepared four standard curves containing each of the con-centrations and tested them by real-time PCR. Repeatability was determined by calculating the %CV (CV = standard deviation/ mean) for all eight replicates of a single concentration. Intermediate precision was determined by comparing the average C_t values for replicates from each individual to each other.

Limit of Reproducible Detection (LOD)

For each assay, the lowest standard curve concentration that allowed eight of eight replicates to be detected with a cycle threshold under 40 and within 2.0 C_t values of each other was considered the limit of reproducible detection (LOD). To con-firm, two individuals each prepared 20 replicates of the LOD concentration and tested them by real-time PCR on separate plates to generate a total of 40 replicates. Repeatability and inter-mediate precision were determined as previously described.

Inclusivity Testing

The inclusivity of each assay was determined by testing the pathogen-specific primers against nucleic acids extracted from panels containing multiple strains of the target pathogens (Table 2). *P.s. tomato* strains were isolated from tomato in 11 countries, strains of WSMV were isolated from hosts in Australia and eight U.S. states, and strains of *X. fastidiosa* originated from four U.S. states. Tests were carried out at a DNA concentration of 100 pg per reaction volume with three replicates per strain.

Exclusivity Testing

The exclusivity of each assay was assessed by testing the pathogen-specific primers against three panels of nucleic acids. Panels included: a multispecies plant panel consisting of DNA extracted from a range of species chosen for their economic importance or placement in diverse taxa (Table 3), a multispecies animal panel consisting of DNA from a range of species chosen for their economic importance and the likelihood that an animal in this group would be found in association with agricultural environments (Table 4), and a near-neighbor microbe panel consisting of nucleic acid extracted from phylogenetic and environmental neighbors of each target pathogen (Table 5). Tests were carried out at a DNA concentration of 100 pg per reaction with three replicates per species.

Positive Control Plasmid Development

For each assay, a plasmid containing the target sequence of each pathogen with an inserted AvaI restriction site was pro-duced commercially (Integrated DNA Technologies, San Diego, CA). The presence of the added restriction site allows the ampli-con from the positive control to be easily distinguished from the native amplicon by restriction enzyme digestion.

Positive Control Plasmid Sensitivity

The sensitivity of each assay was determined utilizing plasmid standard curves. Ten-fold serial dilutions of plasmids, containing from 100,000 target copies to one target copy, were prepared and tested by two different individuals on different days. The repeatability and intermediate precision of each assay were deter-mined as previously described.

Positive Control Plasmid Restriction Enzyme Digestion

To ensure that positive controls could be easily distinguished from native signature, amplified products from genomic or copy DNA and cloned positive control plasmids were subjected to digestion with AvaI restriction enzyme. Reaction volumes of
50 lL contained 1 lL of AvaI enzyme (New England Biolabs, Ipswich, MA), 5 lL of NEBuffer4 (New England Biolabs), and 1 ng of pathogen DNA or 44 lL of the positive control at a concentration of 20,000 copies/lL. Reactions were held at 37°C for 1 h. The

resulting fragments were visualized by gel electrophoresis using a 2% agarose gel supplemented with 0.1 lL/mL of SYBR Safe DNA Gel Stain (Invitrogen).

Results

Linearity and Range

To establish the linear range of each assay, two analysts collected data from testing of serial dilutions of pathogen DNA. The assays produced linear amplification of target DNA from 10 ng to 10 fg for P.s. tomato, from 1 ng to 10 fg for *X. fastidiosa*, and from 1 ng to 100 fg for WSMV. For each assay, the % CV for all replications of a single DNA concentration were below 5.0, indicating that the assays are sufficiently repeatable for NBFAC testing needs. Additionally, average C_ts for each concentration obtained by each analyst differed by fewer than 2.0 C_t values, indicating that the assays display good intermediate precision (Table 6). Limit of Detection

For each assay, the lowest standard curve concentration that allowed eight of eight replicates to be detected with a cycle threshold below 40 and within 2.0 C_t values of each other was considered the limit of reproducible detection (LOD). The presumptive LODs for each assay were 100 fg of gDNA for the P.s. tomato assay, 10 fg of gDNA for the *X. fastidiosa* assay, and 100 fg of cDNA for the WSMV assay. This testing confirmed the LOD for each assay. Comparison of average C_t values between individuals and %CVs below 5.0 for each assay demonstrated that all three assays are both repeatable and

precise at their respective limits of detection (Table 7).

Inclusivity Testing

The inclusivity of each assay was determined by testing the specific primers against a panel of DNA from multiple strains of the target pathogen (Table 2). Each assay was able to detect all inclusivity panel members.

Exclusivity Testing

The exclusivity of each assay was determined by testing the pathogen-specific primers against three panels of DNA: a multispecies plant panel, a multispecies animal panel, and a near-neighbor microbe panel (Tables 3, 4, and 5). The *P.s. tomato* and X. *fastidiosa* specific primers did not amplify any DNA from phylogenetic or environmental neighbors. The WSMV specific primers did not detect any members of the plant or animal panels; however, they did produce amplification when tested against cDNA from the closely related viruses Wheat soil-borne mosaic virus and Triticum mosaic virus.

Positive Control Plasmid Sensitivity

The sensitivity of each assay was determined using plasmid standard curve preparations. The *P.s. tomato* and WSMV assays routinely detected 100 copies and one copy of their respective plasmid controls, while the *X. fastidiosa* assay could detect consistently only 1000 plasmid copies. Comparison of average C_t values between analysts and %CVs

below 5.0 for each assay demonstrated that the assays are repeatable and precise down to 100 plasmid copies for *P.s. tomato*, 1000 plasmid copies for *X. fastidiosa*, and one plasmid copy for WSMV (Table 8).

Positive Control Plasmid Restriction Enzyme Digestion

To ensure that the mutagenized positive control plasmids could be distinguished easily from native signature, amplicons from PCR performed on DNA and plasmid preparations were subjected to digestion with the AvaI restriction enzyme. For all three assays, digestion of amplicons from plasmid DNA resulted in smaller fragments that could be easily distinguished from native signature. Typical results are shown (Fig. 1). Discussion

The 2001 case of intentional dissemination of *Bacillus anthracis* through the U.S. mail prompted significant interest in the nation's capabilities in microbial forensics. In addition to concerted efforts directed at human pathogen forensics, there was also a recognized need for forensically stringent and valid detection and identification assays for high consequence agricultural pathogens (3,22). In the event of an intentional, criminal introduction of a plant pathogen into a U.S. crop, forest, or range-land, forensic tools will be needed for their investigation. The assays must be validated to ensure that their results are reliable and defensible in a court of law (23).

In this study, we developed real-time PCR assays and assay controls for the model plant pathogens *P.s. tomato, X. fastidiosa*, and WSMV. The assays were subjected to rigorous validation for suitability in microbial forensics investigations.

The assays were able to detect consistently minute quantities of nucleic acid, with detection limits of 100 fg of gDNA, 10 fg of gDNA, and 100 fg of cDNA for *P.s. tomato, X. fastidiosa*, and WSMV, respectively. Furthermore, all assays dis-played linear amplification of DNA standard curve preparations, signifying that they may be used both qualitatively and quantitatively.

Results obtained from inclusivity testing of the assays indicate that each assay is able to detect multiple strains or subspecies of its target pathogen. This factor is especially important for the *X. fastidiosa* detection assay because subspecies of the pathogen differ significantly, occurring in different geographic areas, dis-playing different host ranges, and inducing varied symptoms in infected plants (9,11–13). Inclusivity of the assays ensures that they can be used for pathogen detection and identification in various geographic regions. Exclusivity of assays used in microbial forensic investigations involving agriculture is important to ensure that primers do not react with environmental nucleic acids, leading to false-positive results. The assays developed in this work were all found to be exclusive to their target pathogens. The pathogen-specific primers showed no amplification when tested against DNA extracted from various plant and animal species that are likely to be found in association with agricultural set-tings. The *P.s. tomato* and *X. fastidiosa* assays did not detect DNA from near-neighbor organisms either; however, the WSMV specific primers produced amplification when tested against cDNA from *Wheat soilborne mosaic virus* (WSBMV) and *Triticum mosaic virus* (TriMV). As the viral nucleic acids were extracted from field-collected, naturally infected plant tis-sue, we believe that the latter results are most likely due to natural co-infection of the host with

WSMV and the closely related panel virus. WSMV and TriMV commonly co-infect the same plant, and WSBMV may occur together with WSMV (24,25).

The positive control plasmids developed in this study contained the target sequence for a given pathogen-specific primer pair and an added AvaI restriction site. Cleavage of the positive control amplicon into 2 fragments, distinguishing it from pathogen nucleic acid, ensures that a positive assay result is due to the presence of native signature in the sample and not from contamination with positive control material.

As the nation's microbial forensics capabilities continue to increase and the capabilities for plant pathogen forensics expand, additional assays will need to be developed and validated for high consequence and newly emerging plant pathogens. The assay validation procedures detailed in this work provide a framework by which such assays may be developed.

TABLE 1—Inclusivity panels used in validation of Pseudomonas syringae pathovar tomato, Xylella fastidiosa, and WSMV real-time PCR assays.

Pathogen	Strain	Host	Origin	Source
P.s. tomato	DC3000	Tomato	United Kingdom	C. Bender, Oklahoma State University, Stillwater, OK
	1318		Switzerland	
	Pst26L		South Africa	
	3357		New Zealand	
	2844		United Kingdom	
	RG4		Venezuela	
	880		Yugoslavia	
	1108		United Kingdom	
	2846		Canada	
	30555		Australia	
	CPST 147		Czech	
	JL1035		California, United States	
	TF1		United States	
	IPV-B0		Italy	
X. fastidiosa	Temecula	Grape	California, United States	American Type Culture Collection, Manassas, VA
	200901779		Oklahoma, United States	PDIDL, Oklahoma State University, Stillwater, OK
	200902697			
	200902348			
	200902412			
	200902259			
	C178D			
	TX PD 1		Texas, United States	B. Bextine, The University of Texas at Tyler, Tyler, TX
	TX PD2			
	C121D	Oak	Oklahoma, United States	PDIDL, Oklahoma State University, Stillwater, OK
	CVC 50024	Citrus	Brazil	D. Luster, USDA-ARS FDWSRU, Fort Detrick, MD
	CVC 50031			
WSMV	Sidney 81	Wheat	Nebraska, United States	R. French, University of Nebraska, Lincoln, NB
	Type		Kansas, United States	
	(88)JB		Texas, United States	Great Plains Diagnostic Network Wheat Survey
	CO-17		Colorado, United States	
	UW-81		Wyoming, United States	

(586)ND-9	North Dakota, United States	
CO-7	Colorado, United States	
117	Kansas, United States	
425	Oklahoma, United States	
(71)GC1	Texas, United States	
Kali	Montana, United States	
OSU	Oklahoma, United States	R. Hunger, Oklahoma State University, Stillwater, OK
Alvaro	Australia	AGWEST Plant Laboratories, South Perth, Australia
Franco	Australia	

PDIDL, Plant Disease and Insect Diagnostic Laboratory; USDA-ARS FDWSRU, U.S. Department of Agriculture Agricultural Research Service Foreign Disease-Weed Science Research Unit.

TABLE 2—Primers and probes used for real-time PCR amplification.

Assay	Primer Set	Nucleotide Sequences (5′–3′)
P.s. tomato	Pst-F	TGTGCCCAATACATCCAAGA
	Pst-R	CTCCGTTGTCGCTCACTCTA
	Pst-P	FAM-TTTAGCGCACCTCAACCAAAGCC-TAMRA
X. fastidiosa	Xf-F	TGGGTTTATGTTGGCGATTT
	Xf-R	ACTTTCATGGTGGAGCCTGT
	Xf-P	FAM-CAAGCAGGGGGTCGTCGGTT-TAMRA
WSMV	WSMV-F	GAAACGCTTACAGGTGGGTATT
	WSMV-R	CGCTTCCCTTGGTATTCAAC
	WSMV-P	FAM-TGGGAGAAGGAGCAAGAAAGCACA-TAMRA

F, Forward; R, Reverse; P, Probe.

TABLE 3—Plant exclusivity panel used in validation of real-time PCR assays for Pseudomonas syringae pathovar tomato, Xylella fastidiosa, and WSMV.

Plant	Variety	Common Name	Source
Triticum aestivum	Deliver	Hard red wheat	R. Hunger, Oklahoma State University, Stillwater, OK
Medicago sativa	Vernal	Alfalfa	S. Marek, Oklahoma State University, Stillwater, OK
Hordeum vulgare	Post 90	Barley	R. Hunger, Oklahoma State University, Stillwater, OK
Secale cereale	Maton	Rye	R. Hunger, Oklahoma State University, Stillwater, OK
Avena sativa	Okay	Oat	R. Hunger, Oklahoma State University, Stillwater, OK
Oryza sativa	Drew	Rice	J. Leach, Colorado State University, Fort Collins, CO
Sorghum bicolor	Sugar Drip	Sorghum	R. Hunger, Oklahoma State University, Stillwater, OK
Glycine max	VNS	Soybean	Payco Seeds, Dassel, MN
Zea mays	Kandy Korn	Corn	Ferry-Morse Seed Co., Fulton, KY
Arachis hypogaea	TX 313	Peanut	H. Melouk, USDA-ARS, Stillwater, OK
Gossypium hirsutum	Ac44E	Cotton	C. Bender, Oklahoma State University, Stillwater, OK
Arabidopsis thaliana	Landsberg erecta	Thale cress	Lehle Seeds, Round Rock, TX
Lycopersicon esculentum	Wisconsin 55	Tomato	L. L. Olds Seed Co., Madison, WI
Carya illinoiensis	VNS	Pecan	A. Payne, Oklahoma State University, Stillwater, OK
Prunus persica	Jefferson	Peach	A. Payne, Oklahoma State University, Stillwater, OK
Vitis aestivalis	Cynthiana	Grape	A. Payne, Oklahoma State University, Stillwater, OK
Helianthus annuus	Mammoth Grey	Sunflower	L. L. Olds Seed Co., Madison, WI
Nicotiana tabacum	Samsun NN	Tobacco	J. Verchot, Oklahoma State University, Stillwater, OK
Nephrolepsis exaltata	VNS	Boston fern	Department of Entomology & Plant Pathology, Oklahoma State University, Stillwater, OK
Cladonia rangiferina	VNS	Reindeer moss	Teresa's Plants & More Store, Mulberry, AR

VNS, variety not specified; USDA-ARS, United States Department of Agriculture-Agricultural Research Service.

TABLE 4—Animal exclusivity panel used in validation of real-time PCR assays for Pseudomonas syringae pathovar tomato, Xylella fastidiosa, and WSMV.

Organism	Common Name	Source
Homo sapiens	Human	M. James, Oklahoma State University, Stillwater, OK
Bos taurus	Cow	OADDL, Oklahoma State University, Stillwater, OK
Equus ferus	Horse	OADDL, Oklahoma State University, Stillwater, OK
Odocoileus virginianus	White-tailed deer	OADDL, Oklahoma State University, Stillwater, OK
Canis lupus	Dog	OADDL, Oklahoma State University, Stillwater, OK
Felis catus domesticus	Cat	OADDL, Oklahoma State University, Stillwater, OK
Gallus gallus	Chicken	Food Pyramid, Stillwater, OK
Mus musculus	Mouse	Biochain Institute, Inc., Newark, CA
Oryctolagus cuniculus	Rabbit	Biochain Institute, Inc., Newark, CA
Acyrthosiphon pisum	Pea aphid	J. Dillwith, Oklahoma State University, Stillwater, OK
Musca domestica	House fly	A. Wayadande, Oklahoma State University, Stillwater, OK
Homalodisca vitripennis*	Glassy-winged sharpshooter	PDIDL, Oklahoma State University, Stillwater, OK

OADDL, Oklahoma Animal Disease Diagnostic Laboratory; PDIDL, Plant Disease and Insect Diagnostic Laboratory. *Used in X. fastidiosa assay validation only.

TABLE 5—Near-neighbor exclusivity panels used in validation of real-time PCR assays for Pseudomonas syringae pathovar tomato, Xylella fastidiosa, and WSMV.

Assay	Organism	Source
P.s. tomato	Burkholderia cepacia ATCC 25416	American Type Culture Collection, Manassas, VA
	Campylobacter jejuni ATCC 33291	American Type Culture Collection, Manassas, VA
	Escherichia coli 1472	S. Gilliland, Oklahoma State University, Stillwater, OK
	Erwinia tracheiphila	B. Bruton, USDA-ARS[1], Lane, OK
	Lactobacillus delbruckeii ssp. bulgaricus 3409	S. Gilliland, Oklahoma State University, Stillwater, OK
	Pseudomonas aeruginosa 8830	S. Gilliland, Oklahoma State University, Stillwater, OK
	Pseudomonas fluorescens ATCC 13525	American Type Culture Collection, Manassas, VA
	Pseudomonas syringae pv. maculicola 4326	C. Bender, Oklahoma State University, Stillwater, OK
	Pseudomonas syringae pv. phaseolicola 1448A	C. Bender, Oklahoma State University, Stillwater, OK
	Pseudomonas syringae pv. syringae B728A	C. Bender, Oklahoma State University, Stillwater, OK
	Pseudomonas syringae pv. tabaci	C. Bender, Oklahoma State University, Stillwater, OK
	Ralstonia solanacearum ATCC 11696	American Type Culture Collection, Manassas, VA
	Rhizobium rhizogenes ATCC 11325	American Type Culture Collection, Manassas, VA
	Vibrio parahaemolyticus ATCC 17802	American Type Culture Collection, Manassas, VA
	Xanthomonas vesicatoria ATCC 35937	American Type Culture Collection, Manassas, VA
X. fastidiosa	Burkholderia cepacia ATCC 25416	American Type Culture Collection, Manassas, VA
	Escherichia coli 1472	S. Gilliland, Oklahoma State University, Stillwater, OK
	Erwinia tracheiphila	B. Bruton, USDA-ARS, Lane, OK
	Lactobacillus delbruckeii ssp. bulgaricus 3409	S. Gilliland, Oklahoma State University, Stillwater, OK
	Pseudomonas syringae pv. tabaci	C. Bender, Oklahoma State University, Stillwater, OK
	Ralstonia solanacearum ATCC 11696	American Type Culture Collection, Manassas, VA
	Rhizobium rhizogenes ATCC 11325	American Type Culture Collection, Manassas, VA
	Xanthomonas campestris pv. campestris	C. Bender, Oklahoma State University,

		Stillwater, OK
	Xanthomonas campestris pv. malvacearum	C. Bender, Oklahoma State University, Stillwater, OK
	Xanthomonas campestris pv. nigromaculans	C. Bender, Oklahoma State University, Stillwater, OK
	Xanthomonas vesicatoria ATCC 35937	American Type Culture Collection, Manassas, VA
WSMV	Burkholderia cepacia ATCC 25416	American Type Culture Collection, Manassas, VA
	Colletotrichum graminicola	S. Marek, Oklahoma State University, Stillwater, OK
	High plains virus	Great Plains Diagnostic Network Wheat Survey
	Maize dwarf virus	K. Scheets, Oklahoma State University, Stillwater, OK
	Oat necrotic mottle virus	U. Melcher, Oklahoma State University, Stillwater, OK
	Phytophthora capsici	S. Marek, Oklahoma State University, Stillwater, OK
	Pseudomonas fluorescens ATCC 13525	American Type Culture Collection, Manassas, VA
	Rhizopus stolonifer	S. Marek, Oklahoma State University, Stillwater, OK
	Triticum mosaic virus	Great Plains Diagnostic Network Wheat Survey
	Wheat soil-borne mosaic virus	Great Plains Diagnostic Network Wheat Survey
	Wheat spindle streak mosaic virus	J. Verchot, Oklahoma State University, Stillwater, OK
	Xanthomonas vesicatoria ATCC 35937	American Type Culture Collection, Manassas, VA

USDA-ARS, United States Department of Agriculture-Agricultural Research Service.

TABLE 6—Linearity and range of real-time PCR assays for Pseudomonas syringae pathovar tomato, Xylella fastidiosa, and WSMV.

	P.s. tomato Assay			X. fastidiosa Assay			WSMV Assay		
DNA Concentration (per Rxn)	Average C_t Values (8 Curves)	%CV (8 Curves)	# Reps Detected (8 Curves)	Average C_t Values (8 Curves)	%CV (8 Curves)	# Reps Detected (8 Curves)	Average C_t Values (8 Curves)	%CV (8 Curves)	# Reps Detected (8 Curves)
10 ng	23.18	1.07	8	n/a	n/a	n/a	n/a	n/a	n/a
1 ng	26.55	0.61	8	19.23	0.52	8	24.10	0.86	8
100 pg	29.92	1.39	8	23.17	2.50	8	27.16	0.65	8
10 pg	33.01	0.77	8	26.04	0.57	8	30.40	0.34	8
1000 fg	35.06	1.72	8	29.61	1.25	8	33.94	0.63	8
100 fg	35.98	1.12	8	33.04	0.83	8	36.69	1.95	8
10 fg	40.07	1.20	7	36.38	1.25	8	37.48	4.36	8
1 fg	n/a	n/a	n/a	39.01	0.76	2	37.51	3.44	8

TABLE 7—Limit of detection (LOD) of real-time PCR assays for Pseudomonas syringae pathovar tomato, Xylella fastidiosa, and WSMV.

Assay	Technician	Positive Samples (Out of 20)	Average C_t Value (20 Reps)	%CV (20 Reps)
P.s. tomato	1	19	36.31	2.58
	2	18	37.97	2.11
X. fastidiosa	1	20	36.88	2.73
	2	20	36.55	3.12
WSMV	1	19	37.72	1.24
	2	20	35.88	1.52

TABLE 8—Linearity and range of real-time PCR assays on positive control plasmids.

	P.s. tomato Assay			X. fastidiosa Assay			WSMV Assay		
Plasmid Copies (per Rxn)	Average C_t Values (8 Curves)	%CV (8 Curves)	# Reps Detected (8 Curves)	Average C_t Values (8 Curves)	%CV (8 Curves)	# Reps Detected (8 Curves)	Average C_t Values (8 Curves)	%CV (8 Curves)	# Reps Detected (8 Curves)
100,000	24.58	1.99	8	28.40	1.85	8	26.73	1.61	8
10,000	28.15	2.98	8	32.04	3.39	8	30.22	1.09	8
1000	31.84	2.40	8	35.51	1.66	8	33.68	1.12	8
100	36.15	1.47	8	38.73	1.28	6	36.52	1.92	8
10	38.58	4.32	6	–	–	0	38.28	3.94	8
1	–	–	0	–	–	0	37.93	1.53	8

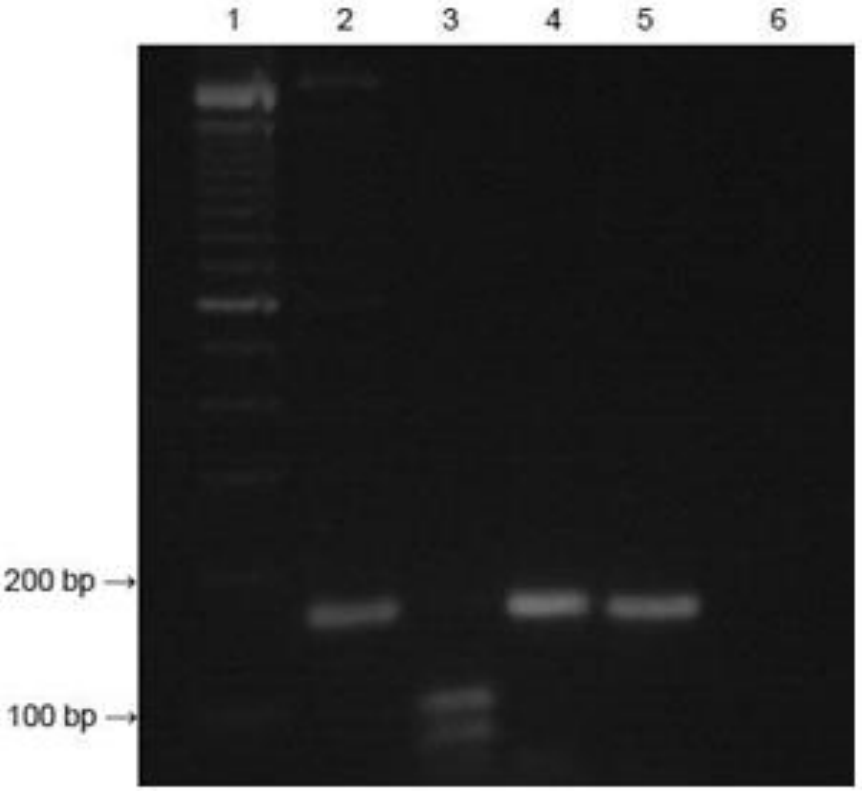

FIG. 1—Agarose gel analysis of AvaI digestion of WSMV positive control and cDNA amplicons (2% agarose gel stained with SYBR Safe DNA Gel Stain [Invitrogen, Carlsbad, CA]). Lane 1, 100 bp DNA ladder (Invitrogen, Carlsbad, CA); lane 2, WSMV positive control amplicon – undigested; lane 3, WSMV positive control amplicon – digested; lane 4, WSMV cDNA amplicon – undigested; lane 5, WSMV cDNA amplicon – digested; lane 6, sterile water – digested.

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CHAPTER IV

Discrimination among *Fusarium proliferatum* strains using inter-simple sequence repeats (ISSRs) and simple sequence repeats (SSRs)

Abstract

The plant pathogen *Fusarium proliferatum* has a wide host range and is present worldwide. The fungus can contaminate grains by producing mycotoxins, which, if ingested, can cause harm to animals and humans. In 2008, an outbreak of salmon blotch of onions, caused by *F. proliferatum*, was detected in southern Israel. The distribution and source of the fungus in Israel were unknown. Inter-simple sequence repeats (ISSRs) and simple sequence repeats (SSRs) were used to characterize populations and discriminate among isolates of the fungus. Seven *F. proliferatum* isolates collected in Israel, Germany, Austria and North America, from cucumber, onion, garlic, maize, asparagus, and salt cedar, were screened using five previously published ISSR primers. Based on the ISSR assays, seventeen SSR primers were designed and tested on ten isolates of *F. proliferatum* from the three countries and the six plant hosts. Six SSR primers consistently amplified single bands from the DNA of each isolate with allele numbers ranging from 6 to 9, depending on the primer. The data demonstrate that these

primers are useful for *F. proliferatum* strain discrimination and that they are applicable also to other species of *Fusarium*.

Introduction

Fusarium proliferatum (Matushima) Nirenberg 1976, a fungal plant pathogen in the phylum Ascomycota, is present worldwide and has a wide host range of 75 plant species, including both monocots and dicots; however, the fungus causes disease in only about half of them (Proctor et al. 2010). Diseases caused by the fungus include rots, diebacks, blights and wilts, and known hosts include onion, mango, wheat, maize, asparagus, palm, pine, and rice (Proctor et al. 2010). The fungus can also produce mycotoxins, such as fumonisins, which pose a health risk to humans and animals if ingested. *F. proliferatum* is also pathogenic to many prairie grasses, where mycotoxins may impact grazers such as bison, elk, and others (Leslie et al. 2004).

In 2008, *F. proliferatum* was isolated from white onions in Yotvata, in southern Israel (Isack et al. 2014). The fungus produces pigmented spores, which appear as salmon-colored blotches on the outer scales of mature bulbs of white onion cultivars. *F. proliferatum* can be isolated from both the outer scales and the internal tissues of white onion bulbs. It rarely causes visible signs or symptoms on yellow and red onion cultivars but can be isolated from the surfaces and the internal tissues of both sets and mature bulbs of such cultivars; albeit at much lower frequencies. Severe colonization by the fungus can lead to bulb rot, rendering the onion unmarketable.

Onion production in Israel begins with the planting of seeds in onion set production fields located in northern Israel, where rainfall is plentiful, followed by the harvesting of young

onion sets that are shipped to arid southern Israel, where they are planted in irrigated fields (Gamliel, personal communication). At maturity, the bulbs are harvested, sorted at packing houses and then sold. Only about 1% of the onions produced in Israel are white cultivars, the majority being yellow and red cultivars. Even though white onions are a slim percentage, a grower can suffer up to 100% crop loss in these cultivars from salmon blotch (Gamliel, personal communication).

F. proliferatum is genetically diverse (Alizadeh et al. 2010), as estimated from the occurrence of vegetative compatibility groups (VCGs), into which fungal isolates from different hosts were separated based on successful pairings. Restriction fragment length polymorphisms (RFLPs) have been used to assess the genetic diversity of *F. proliferatum*. For example, by examining the RFLP profiles of mitochondrial DNA (mtDNA) of 184 isolates, 16 haplotype groups were resolved (Laday et al. 2004). The largest of these, haplotype group I, contained 103 isolates from 7 countries, of which 71% were from maize. These data suggest that there are high levels of genetic variation among *F. proliferatum* populations from multiple hosts, as well as from different geographical locations. In another study, however, the genetic diversity of *F. proliferatum* from a single host, asparagus, evaluated by PCR-RFLP fingerprints, was not correlated with geographical location (von Bargen et al. 2009).

Amplified fragment length polymorphisms (AFLPs) also have been used to characterize the genetic diversity of *F. proliferatum.* After Neumann and Backhouse (2004) examined a population of *F. proliferatum* strains isolated from *Livistona mariae* palms in Finke Gorge National Park, Northern Territory, Australia, they reported genetic variation of strains isolated from a natural ecosystem. Fourteen *F. proliferatum* and *F. verticillioides*

isolates from maize, characterized using AFLPs, could be differentiated based on the polymorphic DNA fragments, which ranged in number from 28 to 51, depending on the primer combination (Visentin et al. 2009). AFLPs are sufficiently informative to distinguish *F. proliferatum* from other *Fusarium* species pathogenic to the same host; for example, isolates of *F. oxysporum* and *F. proliferatum* could be distinguished from each other, but the ten *F. proliferatum* isolates had very similar AFLP patterns (Galvan et al. 2008). Characterization of the genetic variability of fungal isolates from different plant hosts and geographical locations will facilitate a better understanding of the species' evolutionary history from a population biology perspective.

Chandra et al. (2011) reviewed the application of molecular markers such as microsatellites (short [2-6 bp] genetic elements present in eukaryotic genomes) for studying the population biology of *Fusarium* species. Inter simple sequence repeat (ISSR) markers, generated by single-primer polymerase chain reaction (PCR), are short repetitive sequences located between microsatellite loci (Wolfe, 2005). ISSRs can be amplified from a variety of eukaryotes and prokaryotes (Zietkiewicz et al. 1994) to provide a fingerprinting application to assess genetic diversity for taxonomic and phylogenetic studies of a wide range of organisms including *F. proliferatum* (Bayraktar and Dolar, 2011). Simple sequence repeats (SSRs) offer some advantages, such as high reproducibility and high variability among closely related species. SSRs have been described and used for other *Fusarium* species, including, but not limited to, *F. verticillioides*, *F. graminearum*, and *F. solani* f. sp. *pisi* (Ren et al. 2012, Singh et al. 2011, Xiang et al. 2012).

The aim of this study was to develop and validate the use of SSR primers for assessment of the genetic diversity of *F. proliferatum* from different regions and plant hosts. To our knowledge, this is the first application of SSR primers for assessment of the diversity of *F. proliferatum*

Materials and Methods

Fusarium spp. *cultivation and storage*

Fusarium proliferatum isolates used in this study, and their sources, are shown in Table 1. Isolates YO3, YO4, LC29, BG37, YO9, LO11, LOS15 and LO14, from onions grown in Israel, were provided by A. Gamliel (Volcani Institute, Bet Dagan, Israel). Isolates 212S, 231S, 227S, 510S and 223S, from infected asparagus grown in Germany and Austria, were provided by H. Dehne (University of Bonn, Bonn, Germany). Isolates 582 and 2233, from infected maize in the United States, were provided by John Leslie (Kansas State University, USA). Isolates, provided on agar plugs, were transferred to potato dextrose agar (PDA) and incubated at 28°C for 5-7 days. For long term storage, the isolates were grown on PDA plates covered with sterile filter paper for 7-10 days at 28°C and harvested by removing the colonized papers and placing them into sterile 4 oz Whirlpaks (Nasco, Fort Atkinson, WI) at -80°C. Genomic DNA from *F. thapsinum, F. andyazi, F. subglutinans,* and *F. verticillioides,* which were used for the cross-species amplification SSR assay, were provided by James Stack (Kansas State University).

Lyophilization of F. proliferatum *isolates for nucleic acid extraction*

Mycelial mats were transferred into 100 ml Erlenmeyer flasks containing 50ml of liquid potato dextrose broth (PDB) for 10 days and then harvested on filter paper by vacuum filtration from the PDB. Harvested mats were rinsed with sterile water and blotted dry with sterile filter paper before being placed into a 15 ml conical plastic tube and stored at -80°C until lyophilization. Lyophilized mycelium was stored at -80°C until used for DNA extraction.

DNA extraction

DNA was extracted using the UltraClean® Microbial DNA Isolation Kit from MoBio Laboratories (Carlsbad, CA) with the following modifications. Lyophilized *F. proliferatum* mycelium (0.04g) was placed into a 1.5 ml Eppendorf microfuge tube with 3 (2.3mm) sterile chrome steel beads (Biospec Products, Inc, Bartlesville, OK) and the microbeads supplied in the DNA Isolation Kit. The mycelium was subjected to bead beating in a mini beadbeater (Biosepc Products, Bartlesville, OK) at maximum speed 'homogenize' setting, for 30 seconds. Volumes of 300 µl microbead solution and 50 µl of MD1 solution (both supplied in the kit) were added, and the tubes were vortexed briefly and then heated at 65°C for 10 minutes with a brief vortexing after the first 5 minutes. The solids were pelleted at 10,000 x g for 1 min, and the supernatant was transferred to a clean 2.0 ml collection tube. The rest of the DNA extraction was performed according to the manufacturer's protocol, starting with step 7. DNA was eluted in 50 µl volumes and its concentration and purity quantified using a Nanodrop 2000 (ThermoFisher Scientific, Waltman, MA). DNA was stored at -20°C.

ISSR screening of F. proliferatum

A subset of 7 *F. proliferatum* isolates, representing several regions of origin and hosts, were screened with ISSR primers to identify repetitive DNA sequences (Table 1). Universal ISSR primers 808, 823, 818, 827, and 817 (Biotechnology Laboratory, University of British Columbia) were tested with genomic DNA from the 7 isolates with the following PCR cycle; 95°C for 5 minutes, 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 2 minutes, a final extension at 72°C for 10 minutes, for a total of 35 cycles, before a final hold at 4°C. The PCR products were visualized on a 2% 1X TAE agarose gel with 1X TAE buffer for the presence of amplified, repetitive DNA. The *F. proliferatum* isolates for the SSR analysis were selected to represent several regions of origin and several plant host species to maximize the chances of strain diversity sufficient for detection by the SSR technology.

TABLE 1. *Fusarium proliferatum* isolates from different countries and hosts used for ISSR and SSR testing.

Name	Host	Country	ISSR tested	SSR tested
YO4	Onion	Israel	Y	N
SO42	Onion	Israel	N	Y
YC30	Onion	Israel	N	Y
LO11	Onion	Israel	N	Y
LO14	Onion	Israel	N	Y
LOS15	Onion seed	Israel	Y	N
LC29	Cucumber	Israel	Y	N
BG37	Garlic	Israel	Y	N
582	Maize	USA	Y	Y
2233	Maize	USA	N	Y
223S	Asparagus	Germany	N	Y
212S	Asparagus	Germany	N	Y
227S	Asparagus	Germany	Y	Y
510S	Asparagus	Germany	Y	N
231S	Asparagus	Germany	N	Y

Development of SSRs for F. proliferatum

SSR primers were developed, as described by Glenn and Schable (6), for *F. proliferatum* YO3, which had been isolated from a salmon blotch diseased onion in Israel and shown to produce high levels of the mycotoxin, fumonisin (Gamliel, personal communication). Briefly, YO3 genomic DNA was digested using the restriction enzyme *RsaI* (New England Biolabs, Ipswich, MA). Oligonucletide linkers were ligated to the DNA fragments produced by the restriction and amplified by PCR. Commercially purchased magnetic beads (Dynabeads® M-270 Streptavidin, Life Technologies, Grand Island, NY) were coated according to the manufacturer's protocol with biotinylated oligonucleotides of repeated DNA motifs and mixed with the linker-tagged DNA fragments. After hybridization, the beads were washed twice with 400 µl 2x SSC, 0.1% SDS and subsequently four times with 400 µl 1x SSC, 0.1% SDS, to remove unbound DNA. After a final wash with TLE buffer, the DNAs containing the SSR fragments were collected. After a final PCR to amplify the SSR fragments, the PCR products containing the SSR inserts were cloned into competent *E. coli* cells (supplied with the kit) using a TOPO® TA Cloning Kit for Sequencing (Life Technologies, Grand Island, NY). Colonies were screened for the inserts by colony boil PCR according to the kit protocol. The PCR products were cleaned using ExoSAP (Affymetrix, Santa Clara, CA) and sequenced using the ABI 3730 DNA Analyzer (Life Technologies, Grand Island, NY) at the Biochemistry and Molecular Biology Recombinant DNA and Protein Core Facility, Oklahoma State University Stillwater, OK.

SSR primer design and PCR amplification

DNA sequences were edited using ChromasPro V 1.7.5 software (Technelysium Pty, Ltd) to make a single contig from the forward and reverse sequences. Primers were designed using WebSat (9), with the following primer conditions; primer T_m 60°C, GC% 45-50% and product size, 100-400 bp. SSR primers (Table 2) were designed for clones that had more than five di-nucleotide repeats or more than five tri-nucleotide repeats. One primer having a twelve penta-nucleotide repeat also was designed. Primer thermodynamics were evaluated using the mfold Web Server (18), and primers having secondary structures at the 3' ends, which could inhibit PCR efficiency, were eliminated. PCR amplifications were performed in a 20 µl total mixture of 10.5 µl GoTaq® Green Master Mix (Promega Corporation, Madison, WI), 1.0 µl of each primer (5 µM concentration), 6.5 µl nuclease free water (Promega Corporation, Madison, WI), and 1.0 µl DNA (50ng), with the following PCR program, 94°C for 5 minutes, 94°C for 40 seconds, 55°C for 40 seconds, 72°C for 30 seconds for 35 cycles. A final extension was performed at 72°C for 10 minutes and a final temperature hold was at 4°C.

PCR products were visualized on a 2% agarose gel in 1X TAE buffer electrophoresed at 50 volts for 2 hours. The gels were stained with SYBR® Safe DNA Gel Stain (Life Technologies, Grand Island, NY). The PCR products were cleaned using ExoSAP and analyzed using a 2100 Bioanalyzer Instrument with the DNA 1000 Kit (Agilent Technologies, Santa Clara, CA) to obtain the range of band sizes of each SSR primer for all of the isolates.

TABLE 2. Oligonucleotide primers amplifying 17 loci of the *Fusarium proliferatum* genome.

Name	Motif	Forward/Reverse primer	Tm (°C)	Amplicons (range, bp)	No. of alleles	
SSR16	$(GA)_6$	F 5'-GAGCCTTTGTTGTTGGAGAGAC-3'	55	NA	NA	
		R 5'-TCAGATGAGAGAGGATGGTGAA-3'				
SSR18	$(GT)_5$	F 5'-GAGCTGAAGCAAAACCAACTTC-3'	55	1 (358-382)	8	
		R 5'-GTCAGTGTATGGGAAAAGAGCC-3'				
SSR32	$(CT)_5$	F 5'-ATTCCTAAGAGAGGACGAAGGC-3'	55	1-2 (339-411)	NA	
		R 5'-CTACTTCTGTGTGGATAAACGGC-3'				
SSR34	$(TC)_5$	F 5'-AACTCTTTTCAAGCTCTGGACG-3'	55	NA	NA	
		R 5'-TCACAGGTAATGTCAAGGATGG-3'				
SSR36	$(TC)_{11}$	F 5'-GCGACCATGTTGATTCTGTCTA-3'	55	NA	NA	
		R 5'-ACATTTCCTCGGGGTGAGATA-3'				
SSR37	$(TG)_5$	F 5'-CTTTAGCTGTTTGGTCGTTGTG-3'	55	1-2 (266-275)	NA	
		R 5'-ACCTCGGCTCTTAAATCATACG-3'				
SSR38	$(TCT)_7$	F 5'-GAGCTGAAGCAAAACCAACTTC-3'	55	1 (377-408)	8	
		R 5'GTCAGTGTATGGGAAAAGAGCC-3'				
SSR45	\$(TG)_5$	F 5'-CTTTAGCTGTTTGGTCGTTGTG-3'	55	1 (140-149)	7	
		R 5'-CGGGGAGATCCAAGTTATTCTT-3'				
SSR55	$(AGA)_5$	F 5'-CTGCAAGATAGCAAATAGCGTG-3'	55	NA	NA	
		R5'-GTGGGAGGCTACAATGATATGG-3'				
SSR68	$(TGTGT)_{12}$	F 5'-ATGTTGGATACTTCAGGCAGGT-3'	55	1 (110-149	9	
		R 5'-CGTTTTCTGCTCTCCTTCTCTC-3'				
SSR76	$(CTT)_6$	F 5'-ATTCCTAAGAGAGGACGAAGGG-3'	55	NA	NA	
		R 5'-				

		ATGCCAAGTGCATGATAGTCAG-3'				
SSR81	$(AC)_5$	F 5'-ATAGAGAATCAACAGCGGAAGC-3'	55	NA	NA	
		R 5'-TCTTGAGGAGGAAATGAGAAGC-3'				
SSR84	$(AC)_7$	F 5'-CGTCGATTGAAGTAGGCTGA-3'	55	NA	NA	
		R 5'-GAAAGACTCAAATGTCACGCTG-3'				
SSR86	$(GA)_5$	F 5'-AGAAGAGGCTAAAGGCCAAAGT-3'	55	NA	NA	
		R 5'-TTTCCATCATCCCCATCATC-3'				
SSR92	$(GAT)_5$	F 5'-GGCATCGTTTCTAGGGACTGTA-3'	55	1 (352-368)	9	
		R 5'-AGCTGTCTTCTTTGGGGACTCT-3'				
SSR93	$(GAT)_5$	F 5'-ATTCCGGGTGTTTTCAACTG-3’	55	NA	NA	
		R 5'-GGTTGGCTTACAAGTGATCTCC-3'				
SSR109	$(TTG)_5$	F 5'-TGTGGTTGAGAGGTGGTTATGA-3'	55	1 (390-401)	NA	
		R 5'-GGGGATGAGACCATGTAGAAAA-3'				

NA – Not applicable; primer did not amplify and/or produced multiple bands which made allele determination unclear.

Cross-species amplification of SSR markers

One isolate of each of four *Fusarium* species, *F. verticillioides, F. thapsinum, F. subglutinans,* and *F. andiyazi*, were used to test the transferability of SSR markers designed for *F. proliferatum*. Seventeen SSR primers were tested with all four isolates and the PCR products were separated on a 2% agarose gel as described above.

Results

Development of SSR markers

In initial screening of 7 *F. proliferatum* isolates with UBC ISSR primers the amplicon patterns resulting from primers 808, 827, and 817 had the greatest degree of variability (Figure 1), revealing a high degree of variability among the isolates tested. The repeat motifs specific for these three ISSR primers facilitated the identification of a suitable commercial mix of oligonucleotides having the same motifs for the development of SSR markers. Of 17 SSR primers (Table 2) screened with a subset of 10 *F. proliferatum* isolates from Germany, Austria, Israel, and the United States (Table 1), 8 SSR primers consistently amplified the target DNA.

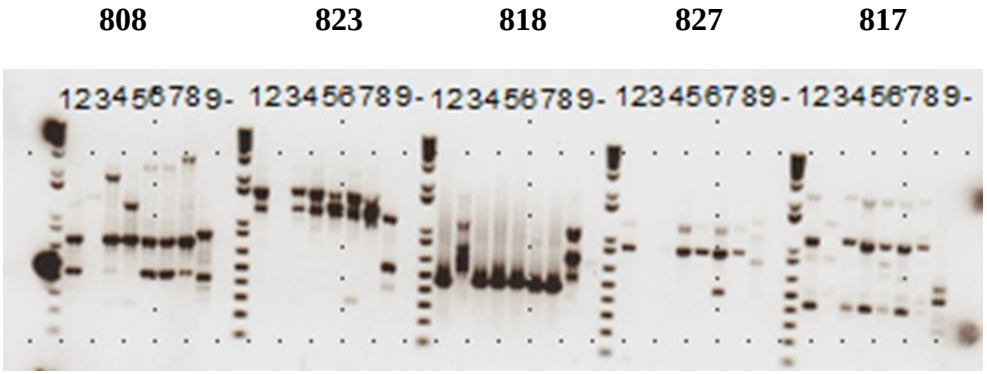


Fig. 1. ISSR amplification of *F. proliferatum* isolates from different countries and hosts.

Genomic DNA of *Fusarium proliferatum* isolates from Germany, North America, and Israel amplified with ISSR primers 808 (AGA)n, 823 (TCT)n, 818 (CAC)n, 827 (ACA)n, and 817 (CAC)n (UBC Primer Set 9, containing universal ISSR primers (Biotechnology Laboratory, University of British Columbia). ISSR primers 808, 827, and 817 produced amplicon patterns with the greatest degree of variability for the isolates tested. Lane 1= 1Kb plus ladder; 2= cucumber (Israel); 3= onion (Israel); 4= garlic (Israel); 5= corn (USA); 6= asparagus (Germany); 7= asparagus (Germany); 8= onion seed (Israel); 9= positive control (*Puccinia emaculata*).

SSR amplification by PCR

Ten *F. proliferatum* isolates from Germany, Austria, Israel, and North America were tested with seventeen SSR primers. SSR primer 68 yielded 9 different amplicon sizes for the 10 total *F. proliferatum* isolates which corresponds to there being (9 alleles), based on the band sizes. YO3 was the most unique isolate from the 10 isolates tested (Figures 2, 3). SSR primer 109 showed the fewest amplicon size differences (6 alleles). SSR primers 18 (8 alleles), 38 (8 alleles), 45 (7 alleles), and 92 (9 alleles) all showed significant levels of amplicon diversity among isolates from different countries as well as from within a country, as was the case when SSR primer 68 was used with the Israel isolates (Figure 1). The other eleven SSR primers tested either did not consistently amplify the DNA, or yielded multiple bands per fungal isolate (data not shown). The ranges of amplicon sizes and of band sizes were determined using a Bioanalyzer. SSR primer 68 had an amplicon range of 110-149 base pairs (bp) (Figure 3). The range of amplicon sizes for the other SSR primers are listed in Table 2.

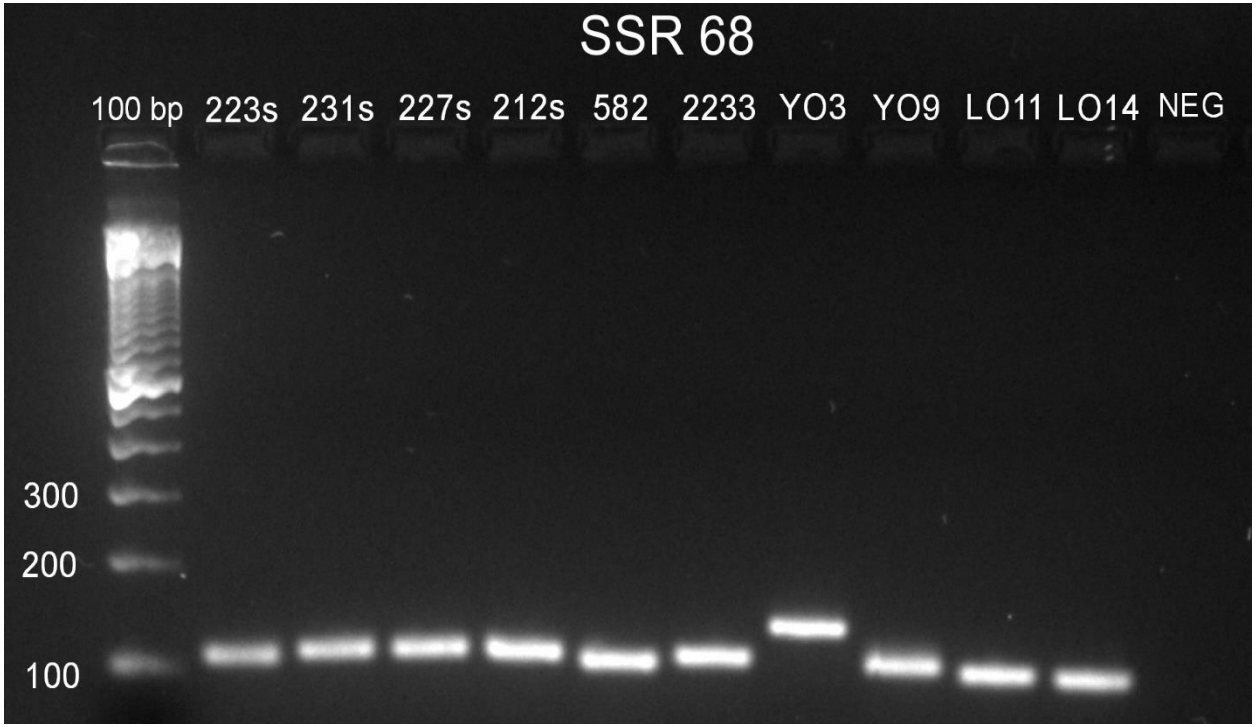
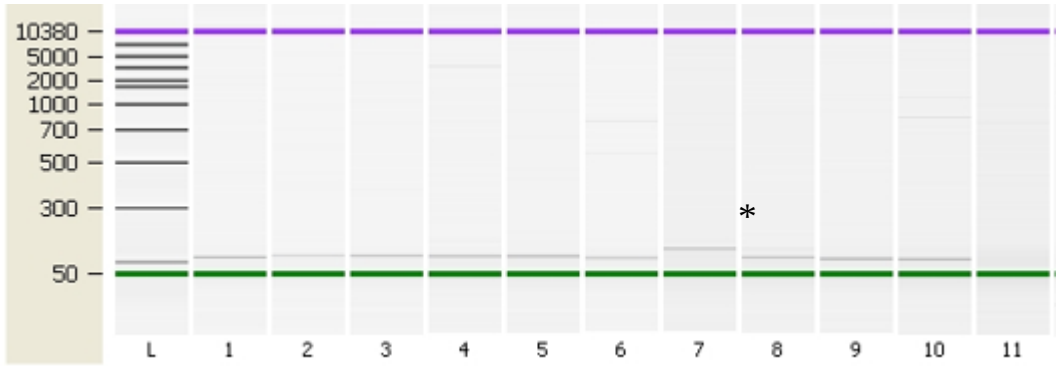


Fig. 2. SSR PCR amplification of *F. proliferatum* isolates from three different countries and three different plant host species using SSR primer 68.

Fusarium proliferatum genomic DNA from asparagus isolates from Germany (223S, 212S), and Austria (231S, 227S) corn isolates from North America (582, 2233), and onion isolates from Israel (YO3, YO9, LO11, LO14) amplified with SSR primer 68. This primer reveals differences among isolates, YO3 being the most unique.



Lane	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**	**L8**	**L9**	**L10**
Isolate	223S	231S	227S	212S	2233	582	YO3	YO9	LO11	LO14
Amplicon size	118 ±5bp	123 ±5bp	122 ±5bp	121 ±5bp	121 ±5bp	115 ±5bp	149 ±5bp	117 ±5bp	112 ±5bp	110 ±5bp

Fig. 3. Bioanalyzer digital gel picture of *Fusarium proliferatum* DNA from isolates from Germany, Austria, North America, and Israel amplified with SSR primer 68. German and Austrian isolates from asparagus are in lanes L1-L4; North American isolates from corn are in lanes L5 and L6; Israel isolates from onion are in lanes L7-L10. Lane L is a 100 bp DNA ladder and lane L11 is a negative control containing no DNA. Band sizes range from 110-149 bp. Isolate YO3 (*) has the greatest band size of the nine isolates.

SSR transferability to other Fusarium *species*

Fusarium verticillioides, *F. thapsinum*, *F. subglutinans*, and *F. andiyazi* were used to test the transferability of the *F. proliferatum* SSR markers. Of the 17 primers, two (SSR 18 and SSR 38) amplified all four species, while six others amplified some but not all of the other species (Table 3). The other 9 primers did not amplify any DNA from any of the four species. SSR primer 93 amplified only *F. thapsinum* and *F. proliferatum* (Table 3).

Discussion

Several DNA based fingerprinting methods have been used to characterize *Fusarium* species. SSR markers have been developed for a number of plants, animals, bacteria, and some fungi (Chandra et al. 2011), but their use for describing genetic variation in plant pathogenic fungi has been limited. PCR-based fingerprinting methods, such as the use of ISSRs and SSRs, have a number of advantages over other technologies. They often take less time and require only minimal pathogen DNA sequence information. In this study, we identified, developed and applied the first SSR markers for *F. proliferatum* strain discrimination. Seventeen SSR primers were tested with ten *F. proliferatum* isolates collected from several different countries and plant hosts; eight primers consistently amplified sequences of all ten fungal isolates and revealed genetic variation among the isolates by variations in amplicon sizes. These data reveal the potential for characterizing large numbers of *F. proliferatum* isolates based on SSR marker analysis.

Our results are similar to those obtained by others who developed and tested SSR markers for *F. oxysporum* and *F. verticillioides*. Using nine SSR markers for *F.*

oxysporum, 5 to 21 nucleotide repeats were identified with 2-15 alleles per locus (Bogale et al. 2005). Four hundred seventy microsatellite loci were identified in *F. verticillioide*s, using a web-based repeats finder, from the full genome sequences of eleven chromosomes (Leyva-Madrigal et al. 2014). These investigators chose eleven loci to design SSR primers and screened 62 *F. verticillioides* isolates to validate their method for identifying microsatellite loci. The range of repeated DNA motifs for each primer was 9 to 35 and the number of alleles for each primer ranged from 7 to 17 (Leyva-Madrigal et al. 2014). In our study, although the greatest number of alleles was identified using SSR primers 68 and 92, all of our SSR primers revealed allele numbers within the same ranges reported by Leyva-Madrigal et al. (2014). The length of primer repeats did not influence the primers' informativeness. For example, a sequence in one locus in the genome of *F. verticillioides* was repeated 35 times and yielded 17 alleles, while another locus having 31 repeats yielded only 8 alleles (Leyva-Madrigal et al. 2014). Similarly, primers SSR 68 and 92 in our study had 12 and 5 repeat units, respectively, and each produced 9 alleles.

The accuracy of allele size resolution is an important consideration in any SSR analysis. The Bioanalyzer 2100, as used in this study to determine allele sizes between 25-100bp, is ± 5bp and the sizing accuracy is ± 10% CV. The SSR resolution reported for *F. verticillioides* was ± 3bp based on the QIAxcel system (Leyva-Madrigal et al. 2014). The Bioanalyzer platform may not have a higher resolution compared to the QIAxcel platform but the resolution between the two is very close but it is advisable to sequence fragments from whatever platform is used to validate the machine's accuracy. Although not done in our study, precise allele sizing is achievable by sequencing the SSR PCR products to

assure that the small base pair differences are due to the variation in repeat number or due to some other circumstance not related to repeat length.

TABLE 3. Cross species amplifications of *Fusarium verticillioides*, *F. thapsinum*, *F. subglutinans*, and *F. andiyazi* with SSR primers.

Fusarium species	Isolate	SSR Primer																
		16	18	32	34	36	37	38	45	55	68	76	81	84	86	92	93	109
F. proliferatum	YO3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. verticillioides	NA	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
F. thapsinum	NA	-	+	-	-	-	+	+	-	-	-	-	-	-	-	+	+	+
F. subglutinans	NA	-	+	-	-	-	-	+	+	-	+	-	-	-	-	+	-	+
F. andyazi	NA	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+	-	+

+ = Successful PCR amplification of DNA with SSR primer

- = SSR primer did not amplify DNA

NA = not applicable, isolate information unknown

Use of the SSR primers described in this paper allows discrimination among *F. proliferatum* isolates from different countries and hosts. The SSR primers also revealed differences among isolates from the same plant host and from the same country, which is seen by the differences in band sizes for the North American isolates from maize and the onion isolates from Israel. The SSR primers are suitable for testing a larger number of *F. proliferatum* isolates from different countries and hosts.

The *F. proliferatum* SSR primers reported here are transferable to other species within the genus *Fusarium*; single isolates of each *F. verticillioides, F. thapsinum, F. subglutinans,* and *F. andiyazi* were amplified using all seventeen SSR primers in this study. Others have reported similar results for SSR primers developed for *F. verticillioides*, which amplified *F. thapsinum, F. nygami, F. andiyazi*, and *F. oxysporum* f. sp. *lycopersici*, (Leyva-Madrigal et al. 2014).

To our knowledge this is the first report of SSR primers designed specifically for *F. proliferatum*. Six SSR primers were polymorphic for the 10 *F. proliferatum* isolates tested in this study. The primers amplify other species of *Fusarium* as well, and could be useful for population studies of this genus.

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CHAPTER V

Simple sequence repeat (SSR) typing of *Fusarium proliferatum* associated with salmon blotch of onions

Abstract

Fusarium proliferatum has a wide host range and is present worldwide. The fungus can contaminate grains and other food products by producing mycotoxins, which, if ingested, can cause harm to animals or humans. In 2008, an outbreak of salmon blotch of onions, caused by *F. proliferatum*, was detected in onion production areas in southern Israel. The distribution and source of the fungus in Israel were unknown. Salmon blotch occurred with increasing severity in subsequent years in the same locations. Several plant and soil substrates were collected from northern Israel, where onion sets are produced, and from southern Israel, where the production occurs, and *Fusarium proliferatum* was isolated from both locations. Isolate recovery was higher for soil samples and onion bulbs collected in southern Israel than from soil and set samples collected in the set production areas in the north. The highest incidences of isolation were from sets and bulbs of white onions (cv. Milky Way), and from soil adjacent to them, in the south. *Fusarium proliferatum* was isolated less frequently from yellow (cvs. Gobi and Ada) and red (cv.

Mata Hari) onion cultivars. DNA fingerprinting of the isolates was performed using simple sequence repeat (SSR)

PCR to characterize populations of the fungus according to the substrate and location from which they were obtained. Phylogenetic analysis, done using the programs GeneAlex, STRUCTURE, BioNumerics, and Numerical Taxonomy System (NTSYS), distinguished the *F. proliferatum* isolates from the Milky Way sets collected in the north from isolates collected from all plant and soil substrates in the south. This finding suggests that those sets are unlikely to have been the source of the *F. proliferatum* strains causing the salmon blotch outbreak. The *F. proliferatum* populations from each of the southern field site soils are similar to one another and to those from the bulbs collected at each of the four southern fields. *Fusarium proliferatum* also was isolated from weeds collected from within the white onion production areas in the Yotvata field. SSR analysis revealed that *F. proliferatum* isolates from volunteer salt cedar are clonal and are indistinguishable from isolates from the Yotvata soil and the 'Milky Way' bulbs. These findings suggest that salt cedar and other volunteer weeds, as well as field soil, could serve as alternative hosts or reservoirs for the fungus, from which inoculum could have moved to the onions and salt cedar.

Introduction

Fusarium proliferatum (Matushima) Nirenberg 1976, a fungal plant pathogen in the phylum Ascomycota, is present worldwide and has a wide host range including 25 monocot, dicot, and conifer species including onion, mango, wheat, maize, asparagus, palm, pine, and rice (Proctor et al. 2010); however, *F. proliferatum* causes disease in only

about half of these plant species. Diseases caused by the fungus include rots, diebacks, blights and wilts, and the fungus can also produce mycotoxins, such as fumonisins, which pose a health risk to humans and animals if ingested. *Fusarium proliferatum* is pathogenic also to many prairie grasses, where mycotoxins may impact grazers such as bison and elk.

In 2002 a new disease of onion, called salmon blotch, appeared in Israel and was attributed to *F. proliferatum* (Gamliel, personal communication). Signs of the pathogen, salmon-colored blotches composed of fungal spores, are easily visible on the outer scales of white onions but are less visible on yellow and red cultivars. If colonization of the fungus is severe, it can lead to bulb rot, rendering the onion unmarketable. The source of the pathogen has not been identified.

Onion production in Israel begins with the planting of seeds in onion set production fields located in northern Israel, where rainfall is plentiful, followed by the harvesting of young onion sets that are shipped to arid southern Israel, where they are planted in irrigated fields (Gamliel, personal communication). At maturity, the bulbs are harvested, sorted at packing houses and then sold. Only about 1% of the onions produced in Israel are white cultivars, the rest being yellow and red. Even though white onions are a small percentage of the total production, a grower can suffer up to 100% crop loss of these cultivars from salmon blotch (A. Gamliel *personal communication*).

It was hoped that identifying the salmon blotch pathogen source could facilitate disease management. Possible sources include the onion seeds, the sets produced in northern Israel, or reservoir plants and/or soils in onion growing regions of southern Israel. In

addition to the traditional epidemiology and disease management challenges created by the emergence and rapidly increasing severity of salmon blotch of onions in southern Israel, the case provided an opportunity to test and validate, in a field setting, newly developed strategies and technologies for forensic investigation of a plant disease.

Plant pathogen forensics combines microbial forensics and plant pathology in a new discipline that enhances capabilities in agricultural biosecurity. Microbial forensics techniques such as DNA fingerprinting using molecular markers, such as simple sequence repeats (SSRs), also known as microsatellites, can be applied to plant pathogens, and have been shown useful for studying the population biology of *Fusarium* (Chandra et al. 2011). Inter simple sequence repeat (ISSR) markers, generated by single-primer polymerase chain reaction (PCR), are short repetitive sequences located between microsatellite loci (Wolfe, 2005). ISSRs have been amplified from a variety of eukaryotes and prokaryotes (Zietkiewicz et al. 1994) and provides a useful fingerprinting approach to assess genetic diversity for taxonomic and phylogenetic studies of a wide range of organisms, including *F. proliferatum* (Bayraktar and Dolar, 2011). SSRs offer some advantages over ISSRs, such as high reproducibility and high variability among closely related species. SSRs have been described and used for other *Fusarium* species, including, but not limited to, *F. verticillioides*, *F. graminearum*, and *F. solani* f.sp. *pisi* (Ren et al. 2012, Singh et al. 2011, Xiang et al. 2012).

The aim of this study was to validate the use of SSR primers for (1) the characterization of *F. proliferatum* populations from different locations and hosts in Israel to assess potential sources of the fungus causing salmon blotch of onions, and (2) their application in a forensic investigation within an agricultural setting.

Materials and Methods

Sampling sites

Four onion production fields in southern Israel were selected for study (Figures 1, 2). All of the fields were planted by the owners (in the case of two commercial fields, Yotvata (designated the investigation field) and Grofit,) or by the Arava Research and Development Experiment Station (ARDES) Manager (two research plots, designated Arava 1 and Arava 2), using normal agronomic practices, with sets of white onion cv. Milky Way, grown from seed in northern Israel. At the Yotvata field, additional rows were planted to onion cultivars Gobi and Ada (yellow) and Mata Hari (red). Each year, onion sets of all cultivars were purchased by and shipped to growers in southern Israel in June or July, and then planted immediately into onion production fields and allowed to grow to maturity (October/November). A variety of crops have been planted in the four experimental fields in previous years; some of these, like maize, are known hosts of the fungus while others, such as potato, are not. Various pre-plant non-chemical (solarization) and chemical (metham sodium) treatments were applied to the fields. Soil solarization was applied in the Yotvata, Grofit, and Arava 1 fields before the sets were planted. A variety of vegetation, including salt cedars, date palms, and weeds, were present in and around the Yotvata field. The Grofit field had little adjacent vegetation, but the Arava 1 and Arava 2 fields were located near other cultivated vegetation within the ARDES. Farm roads extended along some borders of the Yotvata, Arava 1 and Arava 2 fields. At the Yotvata field, only the road separated the field from surrounding salt cedar windbreaks and date palm plantations just beyond.

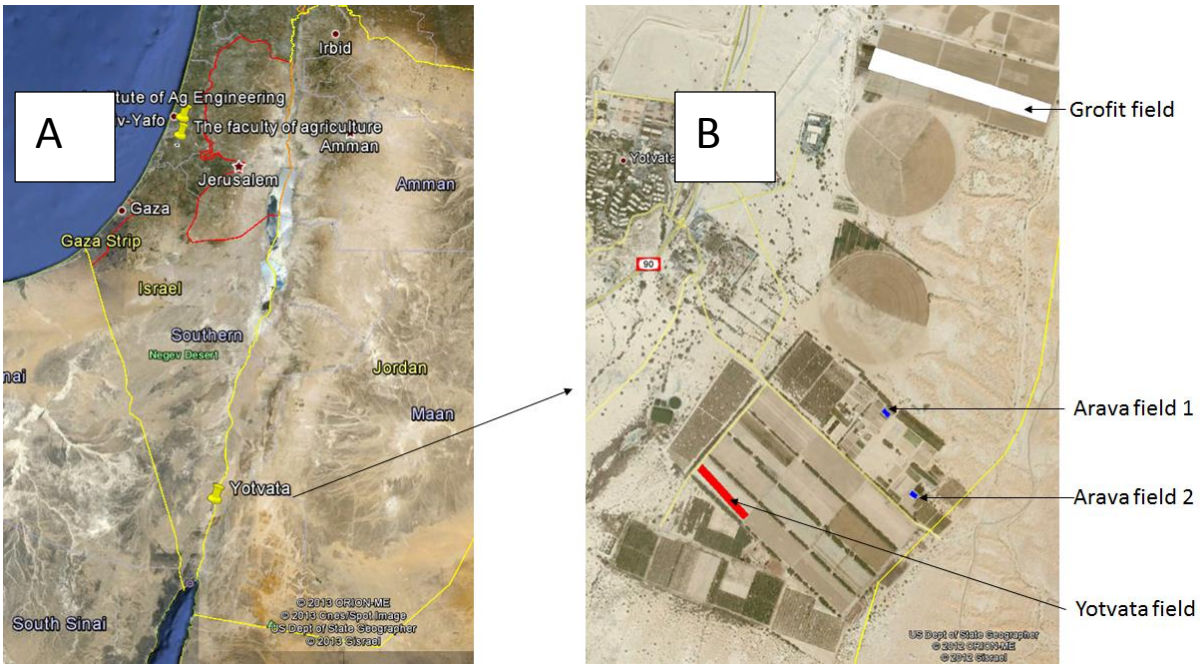

Fig.1. A. Aerial view of Israel showing Yotvata, the location of four field sites for this study. B. Aerial view of two commercial onion fields (Yotvata and Grofit) and two research plots owned and operated by the Arava Research and Development Experiment Station (Arava 1 and 2) are shown (arrows).

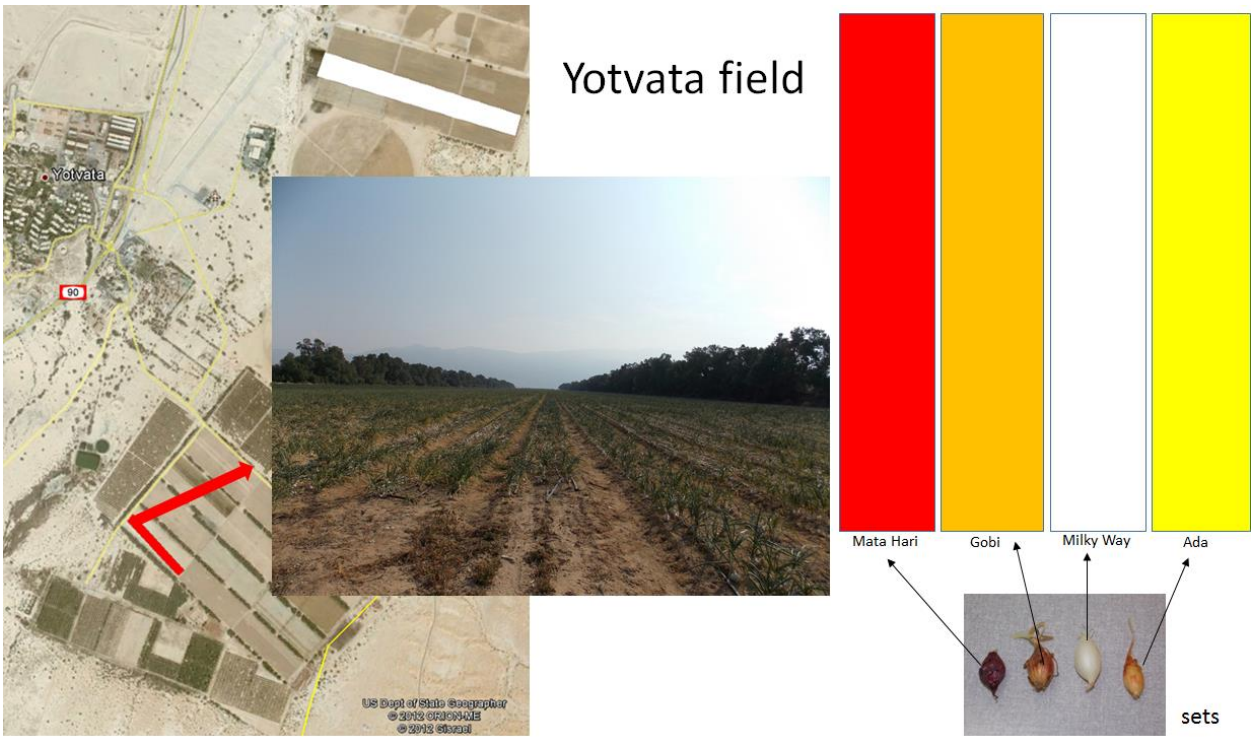

Figure 2. Schematic of the 'Yotvata' field illustrating sets of the red, yellow, and white cultivars planted (referring to salmon blotch in the field).

Sampling of onion bulbs and soils

All plant and soil samples were collected into sterile, individual containers by gloved personnel, as follows:

Yotvata field (Figure 3): Fifty bulbs each of salmon blotch symptomatic white (cv. Milky Way), asymptomatic yellow (cvs. Ada and Gobi), and asymptomatic red (cv. Mata Hari) onions were collected, for a total of 200 bulbs. Symptomatic cv. Milky Way onion bulbs were identified by visible salmon blotches on the outer scales. Bulbs of cvs. Gobi, Ada, and Matha Hari lacked visible salmon blotch symptoms, but their yellow or red pigmentation is likely to mask such signs. Individual bulbs were placed in separate sterile containers, labeled, and placed in a cooler for transport to the laboratory. Soil

samples (a total of 200) were collected from furrows immediately adjacent to each collected bulb, placed into individual plastic bags, labeled and placed in a cooler.

Grofit field (Figure 4): Fifty symptomatic (when present) or asymptomatic white (cv. Milky Way) onion bulbs and fifty adjacent soil samples were collected, labeled, and placed in a cooler as described above.

Arava field 1 (Figure 5): Forty-seven symptomatic (when present) or asymptomatic white (cv. Milky Way) onion bulbs were collected and forty-seven soil samples from adjacent furrows were collected, labeled and placed in a cooler as described above.

Arava field 2 (Figure 6): Forty-two symptomatic (when present) or asymptomatic white (cv. Milky Way) onion bulbs were collected and forty-two soil samples from adjacent furrows were collected labeled and placed in a cooler as described above.

The soil and plant samples were transported to the lab and stored at 4°C until they were processed.

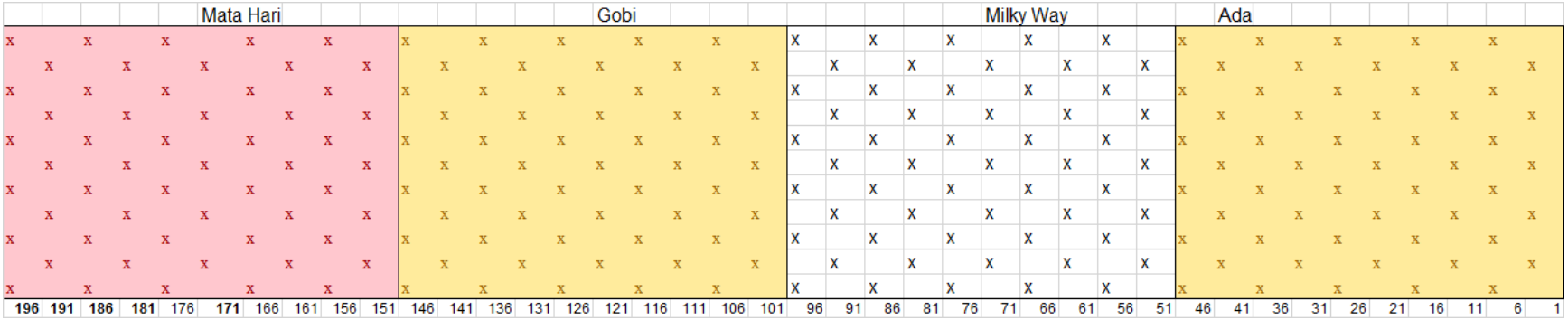

Fig. 3. Yotvata field sampling schematic showing the locations of the four onion cultivars. 'X's indicate where each bulb and soil sample were collected. The sampling locations were 15m apart. Fifty bulbs and soil samples of each cultivar were collected.

X		X		X		X		X	
	X		X		X		X		X
X		X		X		X		X	
	X		X		X		X		X
X		X		X		X		X	
	X		X		X		X		X
X		X		X		X		X	
	X		X		X		X		X
X		X		X		X		X	
	X		X		X		X		X
X		X		X		X		X	
546	**541**	**536**	531	**526**	521	516	511	506	501

Fig. 4. Grofit field sampling schematic showing the location of the onion and soil samples collected. 'X's indicate where each bulb and soil sample was collected. The sampling locations were 15m apart. Fifty bulbs and soil samples of cv. Milky Way were collected.

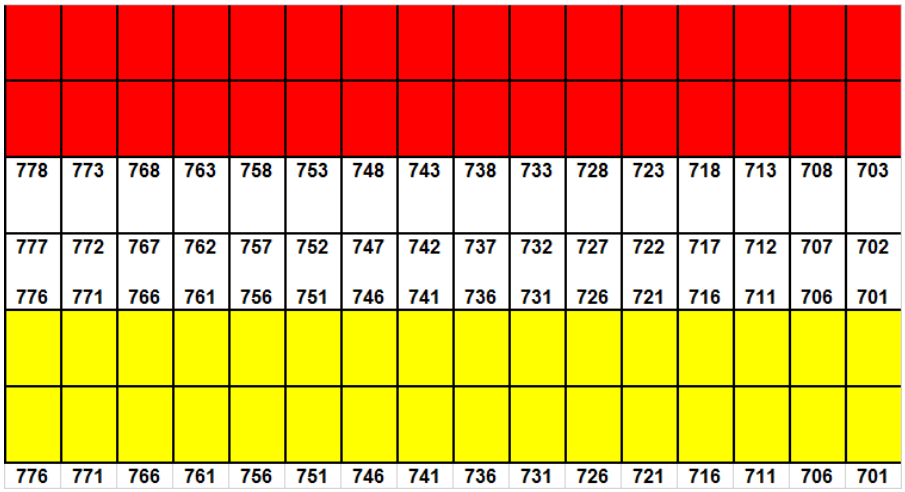

Fig. 5. Arava 1 field sampling schematic showing the location of the onions and soil collected. Numbers indicate where each bulb and soil sample were collected. Forty-eight bulbs and soil samples were collected from onions of cv. Milky Way only. Red and yellow squares represent the locations of rows of red and yellow onion cultivars also present in the field, but not sampled.

	949	944	939	934	929	924	919	914	909	904
	948	943	938	933	928	923	918	913	908	903
952	947	942	937	932	927	922	917	912	907	902
951	946	941	936	931	926	921	916	911	906	901
951	946	941	936	931	926	921	916	911	906	901

Fig. 6. Arava 2 field sampling schematic showing the location of the onion and soil samples collected. Numbers indicate where each bulb and soil sample was collected. Forty-two bulbs and soil samples were collected from onions cv. Milky Way only.

Sampling plant material from salt cedar windbreaks, date palms, and weeds within and adjacent to the Milky Way section of the Yotvata field

Three perimeters, defined based on their distance from the Yotvata field, were sampled in order to assess the geographical distribution of *F. proliferatum* strains present in the field. The first perimeter consisted of salt cedar trees planted as windbreaks north, west, and south of the Yotvata field; the second included date palm trees planted in blocks to the south and west of the Yotvata field; and the third consisted of natural vegetation and weeds growing along highway 90, which connects northern and southern Israel (Figure 7).

Weeds present within the cv. Milky Way bulb field were pulled from the soil with gloved hands, and placed in individual plastic bags. At least 10 samples of each weed species were collected, labeled and placed in a cooler. There were no visible salmon blotch symptoms on any the weeds. The vegetation growing near the highway consisted mainly of woody shrubs. One 6 inch branch cross section was cut from each plant using shears that were sprayed with ethanol after each cutting. The samples were collected, labeled and placed in a cooler.

Figure 7. The sampled windbreaks and field area are located within the yellow rectangle (1). A road separates the date palm plantation to the west from the Yotvata field, but the south plantation directly abuts the windbreak beside the Yotvata field (2). The blue arrow points to the north.

Fungal isolation from onion sets and bulbs

A single batch of apparently healthy onion sets, harvested from a single northern Israel set production field in 2011, was divided into two groups. The larger group was shipped to be planted in the bulb production fields in southern Israel, while the smaller group was sent directly from the set field to the Gamliel laboratory at the Volcani Institute for assessment by fungal isolation. Mature bulbs, collected as described above from the four experimental fields in southern Israel, also were subjected to isolation attempts. In each case, approximately 1 cm^3 of tissue was excised from the onion crown. The tissue was surface sterilized with 3% NaOCl for 1 minute, rinsed twice with sterile distilled water, and placed onto the surfaces of both a semi-quantitative agar (SQA) and a date medium agar (Isack et al. 2014) and held at 28°C for 5 days. Fungal colonies resembling *Fusarium* were hyphal tipped from aerial mycelium using a sterile dissecting needle. The hyphae were placed onto date agar and incubated at 28°C for 5 days. Fungal colonies were examined, using a light microscope (200x), for the presence of polyphialides and chains of microconidia, which are characteristics of *F. proliferatum* (Leslie and Summerell, 2006).

Fungal isolation from soil

Soil samples were collected from two commercial fields, Yotvata and Grofit, and two research plots, Arava 1 and Arava 2, immediately adjacent to each mature bulb collected. Five (~0.05 g) subsamples from each soil sample were each plated, on date agar, and the plates were incubated at 28°C for 5 days. Fungal colonies resembling *F. proliferatum* were identified using a light microscope (200x) and hyphal tipped as described above.

Fungal isolation from onion seeds

Onion seeds, cv. Milky Way, left over from the same lot as those planted in the set production field in the north to produce the sets that were planted later in the four experimental fields (Yotvata, Grofit, Arava 1 and Arava 2), were washed in 50 ml of sterile distilled water to remove surface fungicides and then placed in a sonicating water bath (iUltrasonic, Maplewood, NJ) for 1 minute at its only setting. The seeds were vacuum filtered through cheese cloth to remove the water-fungicide residue, rinsed a second time with sterile distilled water, sonicated for 1 minute, and vacuum filtered as before. After air drying, 20 seeds were plated onto the surface of date agar plates (71 plates total) in a grid pattern and incubated at 28°C for 5 days.

Fungal isolation from non-onion vegetation collected in and around the Yotvata field

To assess the distribution of *F. proliferatum* in the vicinity of the southern onion fields, a variety of weeds, including *Malva nicaeensis* All., *Chenopodium murale* L., *Tamarix aphylla* (L.) Karsten, *Melilotus sulcatus* Desf., *astragalus* spp., *Citrullus colocynthis*, *Avena* spp., and *Phoenix dactylifera* L., were collected from inside the Yotvata field. Salt cedar and date palm seedlings adjacent to the field and a variety of plants growing near a highway that served as the outermost perimeter of the Yotvata field also were collected. A one centimeter-long cross section of the stem of each sample was surface sterilized in (3%) NaOCl for 1 minute, rinsed twice with sterile distilled water, placed onto the surface of a date agar, and incubated at 28°C for 5 days.

Morphological identification of F. proliferatum

Fungal isolates were visualized using light microscopy at 200X to identify polyphilaides and chains of microconidia characteristic of *F. proliferatum* (Leslie and Summerell 2006). Hyphae with these structures were transferred to fresh date agar medium using a sterile dissecting needle and incubated at 28°C for 5 days. Although all of the isolates cultured and used for SSR analysis were identified, based on morphology, as *F. proliferatum*, their identity was not confirmed by another method.

Lyophilization of F. proliferatum *isolates for nucleic acid extraction*

Mycelial mats of fungal isolates were cultured in liquid potato dextrose broth (PDB) (BD Biosciences, San Jose, CA) for 10 days and harvested by vacuum filtration on sterile filter paperThe mats were rinsed with sterile water, blotted dry with sterile filter paper, and stored at -80°C. Lyophilization at -80°C until lyophilization.

DNA extraction

DNA was extracted using an UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) with the following modifications. Lyophilized *F. proliferatum* mycelium (0.04g) was placed into a 1.5 ml Eppendorf microfuge tube with three 2.3mm sterile chrome steel beads (Biosepc Products, Inc, Bartlesville, OK) and the microbeads supplied in the DNA Isolation Kit. The mycelium was subjected to bead beating at maximum speed (homogenize setting), for 30 seconds. Volumes of 300 µl Microbead Solution and 50 µl of Solution MD1 (both supplied in the kit) were added, and the tubes were vortexed briefly and then heated at 65°C for 10 minutes with a vortexing after the first 5 minutes. The solids were pelleted at 10,000 x g for 1 min, and

the supernatant was transferred to a clean 2.0 ml collection tube. The rest of the DNA extraction was performed according to the manufacturer's protocol, starting with step 7. The concentration and purity was quantified using a Nanodrop 2000 (ThermoFisher Scientific, Waltman, MA). DNA was stored at -20°C.

SSR PCR of F. proliferatum *isolates*

Three hundred and nine *F. proliferatum* isolates were amplified using six fluorescent SSR primers (Table 1, Chapter 3) selected from a total of seventeen SSR primers evaluated because they consistently amplified DNA from a variety of *F. proliferatum* isolates from different countries and different hosts (Moncrief, 2014, Chapter 3). The other eleven SSR primers amplified either some or none. PCR reactions were performed in 20 µl total mixtures of 10.5 µl GoTaq® Colorless Master Mix (Promega Corporation, Madison, WI), 1.0 µl of each primer (5µM concentration), 6.5 µl nuclease free water (Promega Corporation, Madison, WI), and 1.0 µl DNA, with the following PCR program, 94°C for 5 minutes, 94°C for 40 seconds, 55°C for 40 seconds, 72°C for 30 seconds for 35 cycles. A final extension was performed at 72°C for 10 minutes and a final temperature hold was at 16°C. Negative controls were performed without the DNA template and positive controls were performed using *F. proliferatum* isolate YO3. The primer characteristics and amplicon ranges are listed in Table 1.

PCR products were cleaned using ExoSAP (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol and the amplicons were submitted for fragment analysis using the ABI 3730 DNA Analyzer (Life Technologies, Grand Island, NY) at the Biochemistry and Molecular Biology Recombinant DNA and Protein Core Facility,

Oklahoma State University Stillwater, OK. To prepare the samples for analysis, 0.4 µl of the size standard LIZ 600 (Life Technologies, Grand Island, NY) was added to 9 µl of Hi-Di Formamide (Life Technologies, Grand Island, NY) along with 1.0 µl of PCR product. The electropherograms were analyzed using PeakScanner software v 1.0 (Life Technologies, Green Island, NY) to determine the amplicon sizes for each of the six SSR primers. The ABI 3730 Analyzer has 98.5% basecalling accuracy.

SSR data analysis

The SSR amplicon sizes produced from each isolate with each primer were recorded in an Excel spreadsheet to create a data matrix. Data were analyzed using the program GeneAlex, which facilitated analysis of molecular variance (AMOVA) and the principal component analysis (PCA) on the 216 *F. proliferatum* isolates. Another population genetics software program, STRUCTURE, was used to define the *F. proliferatum* populations based on probabilities of genetic similarity. BioNumerics 7.1 was used further to characterize the *F. proliferatum* populations based on the number of repeated SSR units present in each isolate. The minimum spanning tree (MST) analysis allowed visualization of the structure of the isolate populations in this study. Finally, the phylogenetics program NTSYS allowed for the creation of a UPGMA dendrogram that positioned the isolates based on the genetic distance between them.

Results

Fungal isolation from onion sets, bulbs, weeds, soil, windbreaks, date palms, and highway vegetation

Fusarium proliferatum was isolated from plant and soil samples in Israel from December to January during 2012-2013 (Table 1). Percentages of samples testing positive for *F. proliferatum* varied with the field, the sample type (bulb vs. soil), and the onion cultivar. The highest *F. proliferatum* isolation frequency (84%) was from the Yotvata field soils in which the 'Milky Way' onions were grown (Figure 8). The isolation frequencies from the soils in which the other three onion cultivars were planted were significantly lower; 44% for Ada, 48% for Gobi, and 56% for Mata Hari (Figure 8). The isolation frequencies from the soils in the other three fields in which 'Milky Way' onions were grown also were lower; 78% for Grofit, 85% for Arava 1 and 45% for Arava 2 (Figures 9, 10, and 11).

Overall, *F. proliferatum* isolation frequencies from onion bulbs were similar to those of the soil samples collected near those same bulbs. For example, presence of the fungus in bulbs of each cultivar in the Yotvata field were similar to those of the corresponding soil samples in the same field; 84% for 'Milky Way', 42% for 'Ada', 70 % for 'Gobi', and 56% for 'Mata Hari' (Table 1). In addition to the forty-two isolates from the 'Milky Way' bulbs, 21 were isolated from cv. 'Ada', 35 from cv. 'Gobi', and 28 from cv. 'Mata Hari'. The number of *F. proliferatum* isolates obtained from the onion sets differed based on cultivar; 48 *F. proliferatum* isolates were cultured from the 50 'Milky Way' sets sampled, but only 3 isolates from 'Gobi' sets and 4 from 'Mata Hari' sets. No isolates were cultured from the 50 'Ada' sets.

Most plant species tested, other than onion, were poor sources of the fungus. Attempts to isolate *F. proliferatum* from the salt cedar windbreaks along the north, south, and east edges of the Yotvata field were unsuccessful, and only one isolate was obtained from 126 total plant samples collected from the areas near the highway. In contrast, the date palm plantations east and south of the Yotvata field harbored *F. proliferatum*, which was cultured from 16 of 117 date palm samples. Onion seeds planted in the northern set fields were devoid of *F. proliferatum;* no isolates were cultured from any of the 1,420 seeds plated onto date agar (Table 1).

TABLE 1. Numbers of *F. proliferatum* isolates cultured from various plant and soil substrates.

Fungal isolations from soil	# positive for *Fp*	# of samples collected	Percentage	**Fungal isolations from sets**	# positive for *Fp*	# of samples collected	Percentage
Yotvata (Milky Way)	42	50	84%	North set field cv. Ada	0	50	0%
Yotvata (Ada)	22	50	44%	North set field cv. Milky Way	48	50	96%
Yotvata (Gobi)	24	50	48%	North set field cv. Gobi	3	50	6%
Yotvata (Mata Hari)	28	50	56%	North set field cv. Mata Hari	3	50	8%
Arava 1	41	48	48%				
Arava 2	19	38	38%	**Fungal isolations from salt cedar windbreaks**	# positive for *Fp*	# of samples collected	Percentage
Yotvata before planting sets	15	50	30%	South of Yotvata field	0	15	0%
Set field soil (northern Israel)	0	15	0%	West of Yotvata field	0	9	0%
Grofit	39	50	48%	North of Yotvata field	0	27	0%
				South of Yotvata field	0	15	0%

Fungal isolations from bulbs	# positive for *Fp*	# of samples collected	Percentage				
Yotvata (Milky Way)	42	50	84%	**Fungal isolations from date palms**	# positive for *Fp*	# of samples collected	Percentage
Yotvata (Ada)	21	50	42%	East of Yotvata field	5	68	7%
Yotvata (Gobi)	35	50	70%	South of Yotvata field	11	49	22%
Yotvata (Mata Hari)	28	50	56%	**Fungal isolations from weeds within the Yotvata field**			
Grofit	30	50	60%	Within the Milky Way art of the field	47	78	60%
Arava 1	35	48	73%				
Arava 2	19	38	50%				
Fungal isolations from plants near the highway	# positive for *Fp*	# of samples collected	Percentage				
SE of Yotvata field	0	23	0				
NE of	1	26	3.8%				

Yotvata field							
SW of Yotvata field	0	25	0%				
NW of Yotvata field	0	52	0%				
Fungal isolations from onion seeds	# positive for *Fp*	# of samples collected	Percentage				
Leftover seeds not planted in the north set fields	0	1420	0%				

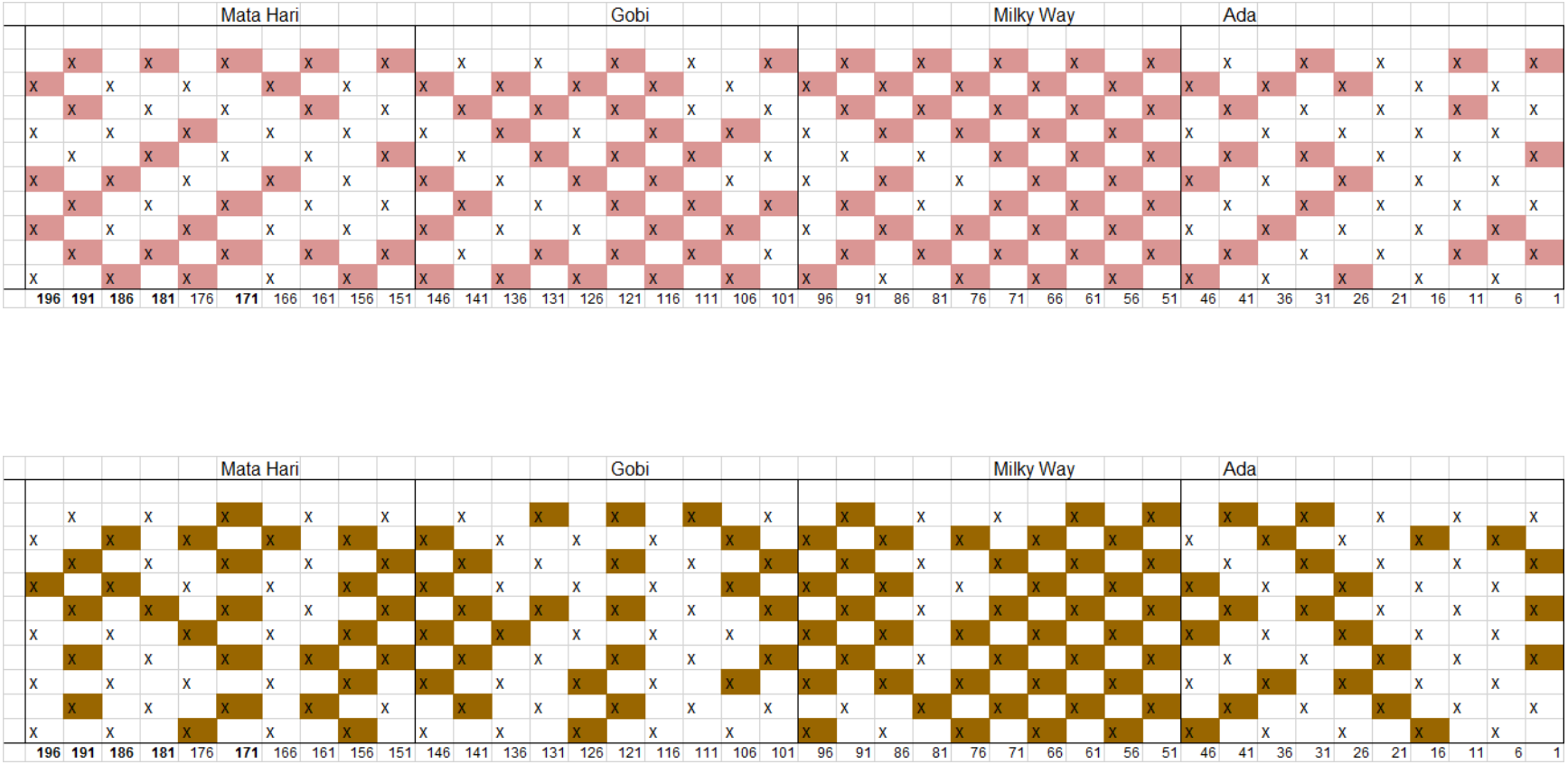

Fig. 8. Schematic of the Yotvata field showing the bulb (pink) and soil (brown) samples that were positive for *F. proliferatum*.

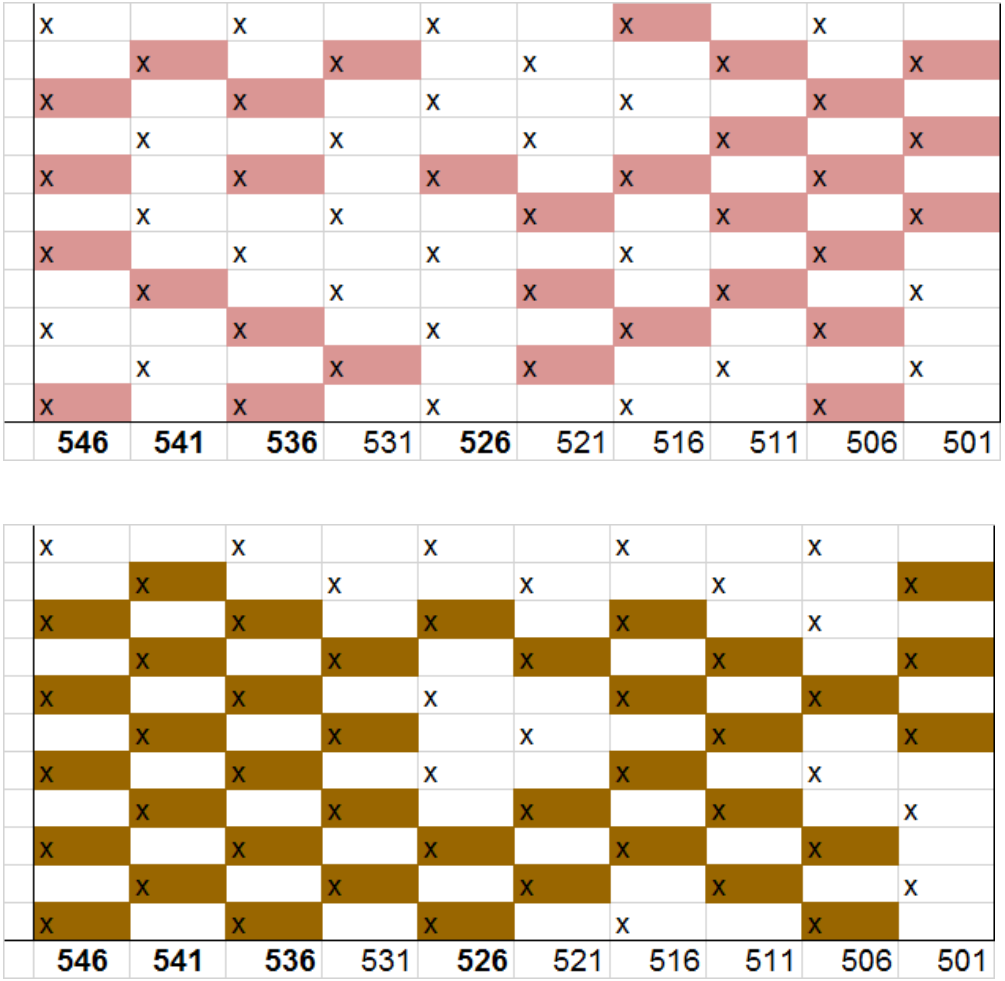

Fig. 9. Schematic of the Grofit field showing the bulb (pink) and soil (brown) samples that were positive for *F. proliferatum*.

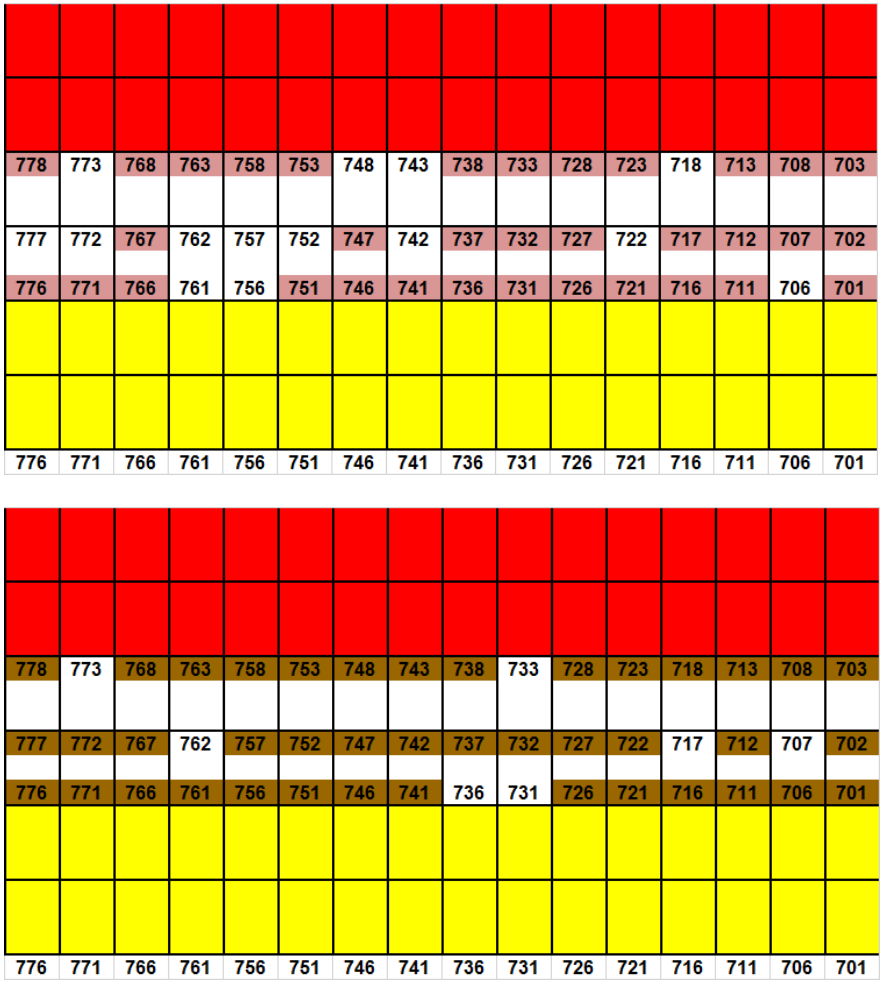

Fig. 10. Schematic of the Arava 1 field showing the bulb (pink) and soil (brown) samples that were positive for *F. proliferatum*.

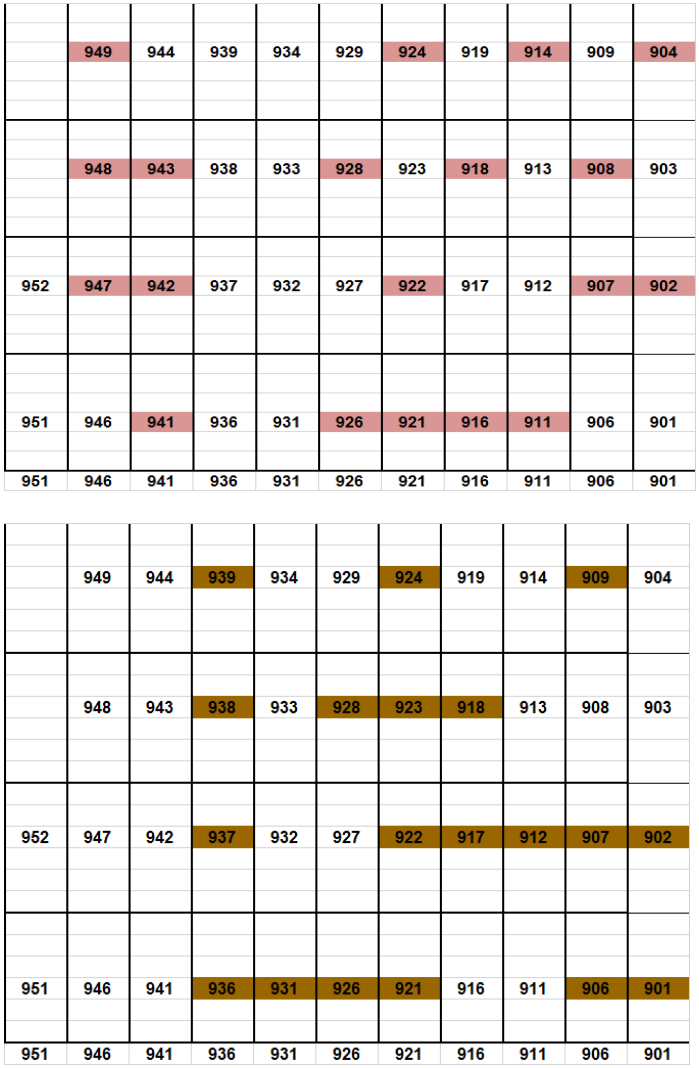

Fig. 11. Schematic of the Arava 2 field showing the bulb (pink) and soil (brown) samples that were positive for *F. proliferatum*.

SSR PCR amplification

Of genomic DNA from 309 Israel *F. proliferatum* isolates tested by PCR using six SSR primers (SSR18, 38, 45, 68, 92, and 109) 216 were amplified consistently with all six primers. DNA of the other 93 isolates either did not amplify with any of the primers, or were amplified with some, but not all, of them. The latter 93 isolates were not included in the phylogenetic analyses. Attempts to repeat those 93 PCR reactions yielded similar results. SSR primer 38 revealed the greatest number of alleles (8), based on differential amplicon sizes ranging from 372-402bp. Primers 68 and 109 each revealed 6 alleles (94-170 and 393-409bp, respectively). Primers 45 and 92 each revealed 5 alleles (140-145 and 348-360bp, respectively). SSR primer 18 yielded only three alleles, (371, 372, and 373 bp).

AMOVA analysis comparing populations

Analysis of molecular variance (AMOVA), used to compare *F. proliferatum* populations from which isolates were collected, describes the amount of genetic variation within and among populations. A total of 216 *F. proliferatum* isolates that were amplified consistently by the six SSR primers were chosen for the analysis. The 216 isolates were grouped in a Microsoft Excel spreadsheet based on the population (substrate) from which they were isolated, and comparisons among them, along with their corresponding PhiPT values (a measure of population genetic differentiation) are shown in Table 3. The populations having the greatest significant diversity, as indicated by their PhiPT, values are:

1) All *F. proliferatum* isolates from the north vs all isolates from the south (PhiPT = 0.655) – The northern *F. proliferatum* population consists only of isolates from the onion sets. The southern population comprises all of the isolates from soils, onion bulbs, date palms, and weeds.

2) All *F. proliferation* isolates from bulbs vs those from date palms (PhiPT = 0.538) – The onion bulbs from all four field locations were significantly different from the isolates from the date palms located south and west of the Yotvata field as well as within the 'Milky Way' portion of the Yotvata field.

3) All *F. proliferatum* isolates from cv. 'Milky Way' bulbs (south) vs those from cv. 'Milky Way' sets (north) (PhiPT – 0.808) – This comparison show the greatest level of diversity.

4) All *F. proliferatum* isolates from sets (all cultivars) vs those from bulbs (all cultivars) (PhiPT = 0.7) – The *F. proliferatum* isolates from the red, white, and yellow sets are genetically different from the bulb isolates, which were derived from a cohort of the same sets.

5) All *F. proliferatum* isolates from cv. 'Milky Way' soil vs those from cv. Ada soil (PhiPT = 0.56) - Despite the fact that cvs. 'Ada' and 'Milky Way' were separated in the Yotvata field by only a single furrow, there is significant diversity among the isolates collected from the soils in which these two cultivars were grown.

Comparisons having lower, but still moderate, levels of genetic diversity include *F. proliferatum* isolates from all onions sets and bulbs vs those from other plant hosts (0.14): salt cedar vs date palms (0.185), salt cedar vs Yotvata field weeds (0.103), and

‘Milky Way’ bulbs vs date palms (0.254). The AMOVA comparisons of the soils from all four field sites show low but significant genetic diversity.

TABLE 2. AMOVA analysis comparing populations of *F. proliferatum* isolates from different locations and substrates in Israel.

Comparison	**PhiPT value**
North vs south	0.655 ***
All sets vs all bulbs	0.7
All onions vs other hosts	0.14
All soils vs plants	0.067
Yotvata soil vs Grofit soil	0.09
Yotvata soil vs Arava 1 soil	0.019
Yotvata soil vs Arava 2 soil	0
Yotvata soil vs soil before sets planted	0
All bulbs vs weeds	0.042
All bulbs vs date palms	0.538 ***
All bulbs vs other hosts not salt cedar volunteers	0.034
All bulbs vs salt cedar volunteers	0.002
All bulbs vs all soils	0
Milky Way soil vs Ada soil	0.56
Milky Way soil vs Gobi soil	0
Milky Way soil vs Mata Hari soil	0
Salt cedar volunteers vs Yotvata weeds	0.103
Milky Way bulbs vs all soils	0.004
Yotvata soil vs salt cedar volunteers	0
Milky Way bulbs vs salt cedar volunteers	0
Milky Way bulbs vs date palms	0.254 ***
Salt cedar volunteers vs date palms	0.185
Milky Way sets vs Milky Way bulbs	0.808 ***

PhiPT values 0= no genetic diversity; 0.05-0.10 = low genetic diversity, but significant; 0.1-0.2 = moderate genetic diversity, significant; 0.2-0.5 = high genetic diversity, significant; >0.5 = great genetic diversity, significant. *** Significant, p value <0.001.

BioNumerics minimum spanning tree analysis

Within the BioNumerics program, the data were analyzed using the multiple locus variable number tandem repeat analysis (MLVA), which compares and characterizes the *F. proliferatum* isolates by the number of SSR repeats present in each. To input the data into the BioNumerics program, the 216 *F. proliferatum* isolates were grouped based on the substrate from which they were isolated. Within the MLVA module, a minimum spanning tree revealed four *F. proliferatum* isolate clusters: A, B, C and D (Figure 12). Each cluster contains isolates from a wide variety of locations (including both northern and southern sites) and substrates (multiple plant hosts as well as soils). The largest cluster (A) comprises 147 *F. proliferatum* isolates, including at least one each from date palms (1 isolate), 'Milky Way' sets (1), 'Mata Hari' sets (1) and 'Gobi' sets (1), weeds (30), soils (63) and bulbs (50). All cluster A isolates are indistinguishable by this analysis. Cluster B, the next largest, comprises 34 isolates from 'Mata Hari' sets (2 isolates), date palms (7), soils (10), weeds (3) and bulbs (10). Within cluster B, two circles contain a mixture of *F. proliferatum* isolates from soil, weeds, and bulbs, and one circle contains 2 isolates from 'Mata Hari' sets and 1 isolate from a 'Milky Way' onion bulb. Cluster C has 16 isolates from 'Milky Way' sets (7 isolates), 'Gobi' sets (2) and soil (2). Cluster D has 20 isolates from 'Milky Way' sets (16 isolates), bulbs (1) and soil (3). Unlike the AMOVA analysis, which provides a PhiPT value to convey a confidence level for the indicated relationships, the minimum spanning tree does not provide a quantitative measure of confidence. However, the grouping patterns resulting from the MLVA analysis are similar to those from the AMOVA analysis.

Overall, the MLVA analysis groups the majority of the sets (grown in northern Israel) in clusters C and D. However, three onion set isolates cluster with the majority of the southern isolates and two set isolates group with cluster B.

The majority of the *F. proliferatum* isolates from the onion bulbs (grown in southern Israel) are in cluster A, where they are indistinguishable from one another. Thirteen onion bulb isolates (including some from each the Yotvata, Grofit, Arava 1 and Arava 2 fields) fall into clusters B and C. All but three of the isolates from weeds inside the 'Milky Way' section of the Yotvata field are in cluster A.

F. proliferatum isolates from the date palm plantations adjacent to the Yotvata field constituted their own cluster, B, except for one isolate that fell into cluster A. The five isolates from the Yotvata soil that was collected in the year before the sets were planted fell into the same cluster A, along with those isolated from the Yotvata soil in 2012. The isolates from the soils of the four fields are predominately grouped together in cluster A, but several are scattered in clusters B, C, and D.

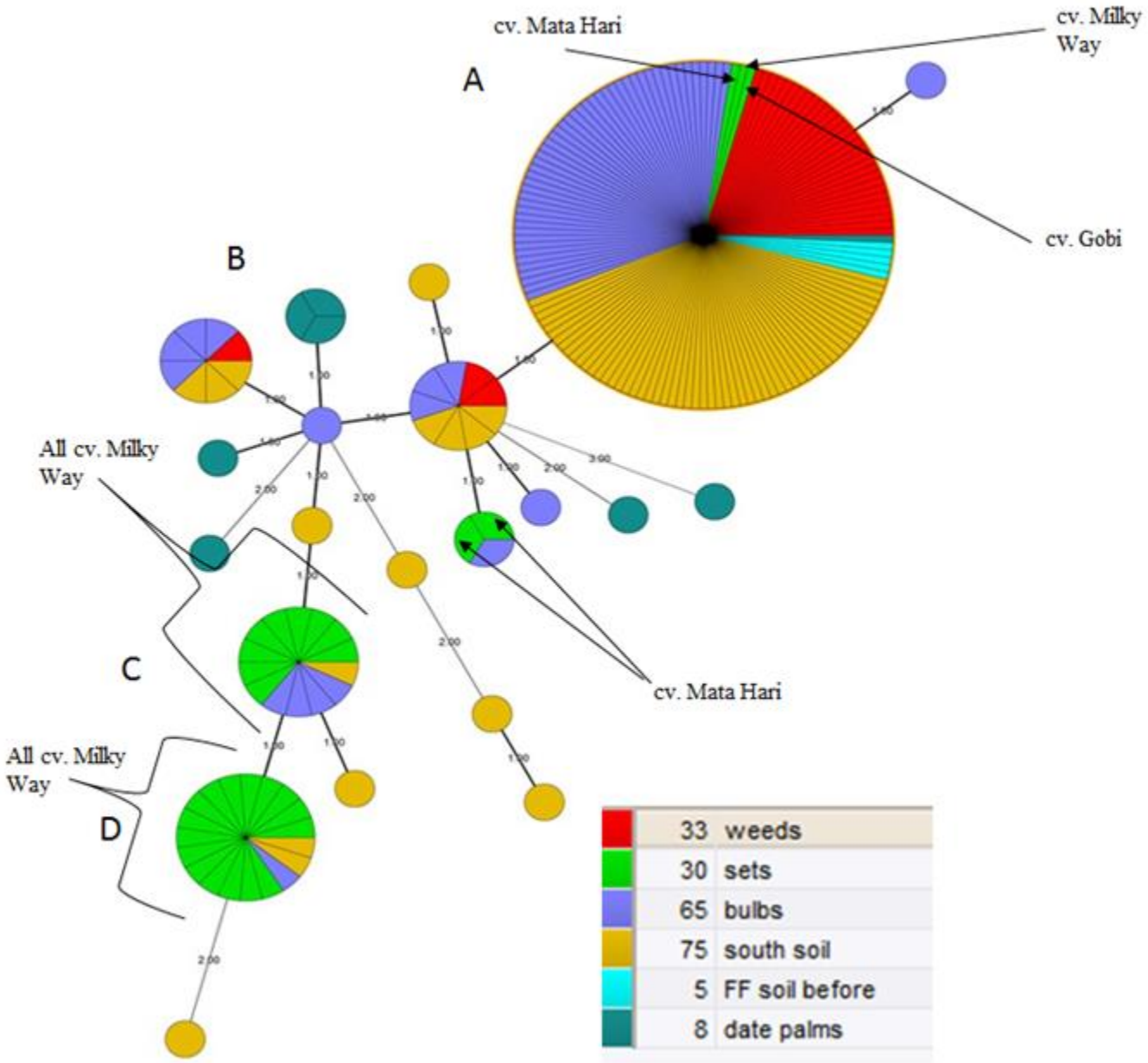
cv. Mata Hari
cv. Milky Way
A
cv. Gobi
B
All cv. Milky Way
C
cv. Mata Hari
All cv. Milky Way
D
33 weeds
30 sets
65 bulbs
75 south soil
5 FF soil before
8 date palms

Figure 12. BioNumerics minimum spanning tree of 216 *F. proliferatum* isolates, showing four major clusters of similarity.

Green: isolates from onion sets (cvs. 'Milky Way', 'Mata Hari' and 'Gobi') grown in northern Israel; yellow: soil isolates from the four bulb production fields in the south; red: isolates from weeds within the Yotvata field; purple: bulb isolates from all four southern fields; light blue: soil isolates from the Yotvata field (collected before the sets were planted); and dark blue: isolates from date palms growing south and west of the Yotvata field.

STRUCTURE analysis

The STRUCTURE analysis compares populations based on their genetic similarities. The 216 *F. proliferatum* isolates used for the AMOVA and the BioNumerics analyses were also used for this analysis. Prior to the STRUCTURE analysis the isolates were identified and grouped together based on the field and the substrate from which they were isolated, yielding14 sub-populations. For each population defined, a probability of genetic similarity is calculated based on the SSR data.

Although 14 sub-populations were inputted into the program, only two populations were recognized in the analysis (Figure 13). Most sets of onion cvs. Milky Way (white) and Gobi (yellow), grown in the north and shown in green in Figure 13, were separated from the southern isolates, shown in red. However, one Milky Way set isolate and one Gobi set isolate showed >99% similarity to the southern population. Unexpectedly, sets of cv. Mata Hari (red) have >95% similarity to the *F. proliferatum* isolates from southern Israel. Isolates from the weeds within the Milky Way section of the Yotvata field are >99% similar to the *F. proliferatum* isolates collected from the bulbs and soil in the Yotvata field. The majority of the isolates from the bulbs and soils in the Yotvata, Grofit, Arava 1, and Arava 2 fields grouped with the second population (red) (Figure 13). There are indications in the STRUCTURE analysis of possible hybridization of *F. proliferatum* isolates from northern and southern Israel, as indicated by a mix of red and green populations. The isolates from the date palms near the Yotvata field are similar to the isolates in population 1 (green), except for a few isolates, which are in either of the two populations.

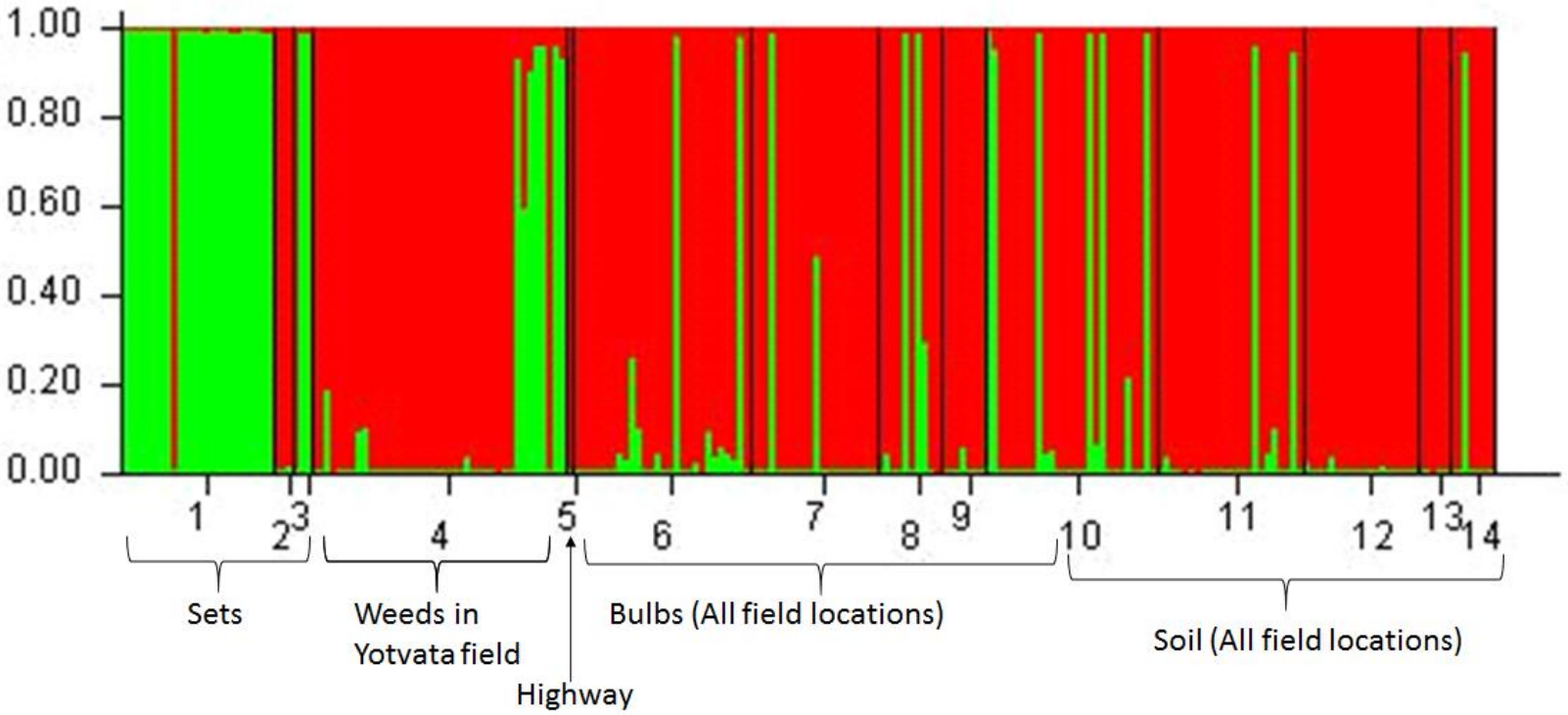

Fig. 13. STRUCTURE analysis of 216 *F. proliferatum* isolates revealing two main populations (green and red). Bars with both green and red colors indicate a mixture of the two populations.

Sub-populations 1 = 'Milky Way' sets (white); 2 = Gobi sets (yellow); 3 = 'Mata Hari' sets (red); 4 = weeds inside Yotvata field; 5 = highway perimeter weed; 6 = Yotvata bulbs (white, red, yellow); 7 = Arava 1 bulbs (white); 8 = Arava 2 bulbs (white); 9 = Grofit bulbs (white); 10 = Yotvata soil; 11 = Arava 1 soil; 12 = Grofit soil; 13 = Yotvata soil before set planting; 14 = Arava 2 soil.

Principal component analysis with GeneAlex

The PCA is a multivariate analysis that identifies patterns in a diverse data set, such as one having multiple loci. The 216 *F. proliferatum* isolates were categorized in the same 14 sub-populations used in the STRUCTURE analysis (Figure 14). Unlike the STUCTURE analysis where the goal was to determine how many populations were observed based on their SSR data, the PCA gives a spatial representation of the isolates and where they cluster together, much like the BioNumerics minimum spanning tree analysis. Overall, the isolates group into two main clusters (blue and green shading), the green cluster comprising the isolates from the north (onion sets) and the two blue clusters comprising the isolates from the south (weeds, bulbs, soil, date palms). The isolates from the white onion (cv. Milky Way) sets clustered together on the PCA plot except for isolate 312, which clustered with the *F. proliferatum* isolates collected from southern Israel (Figure 14, blue shading). The three *F. proliferatum* isolates from the red (cv. Mata Hari) grouped with the isolates from the south. *F. proliferatum* isolates from the yellow (cv. Gobi) onion sets, were scattered around the PCA plot; one isolate clustered with the southern population and two with the northern population. The *F. proliferatum* isolates from the date palms grouped with the southern *F. proliferatum* population, but fell within two clusters. All of the *F. proliferatum* isolates from the southern field soils clustered with the bulbs, weeds, and date palms, except for one group of isolates that clustered closer to the northern *F. proliferatum* population.

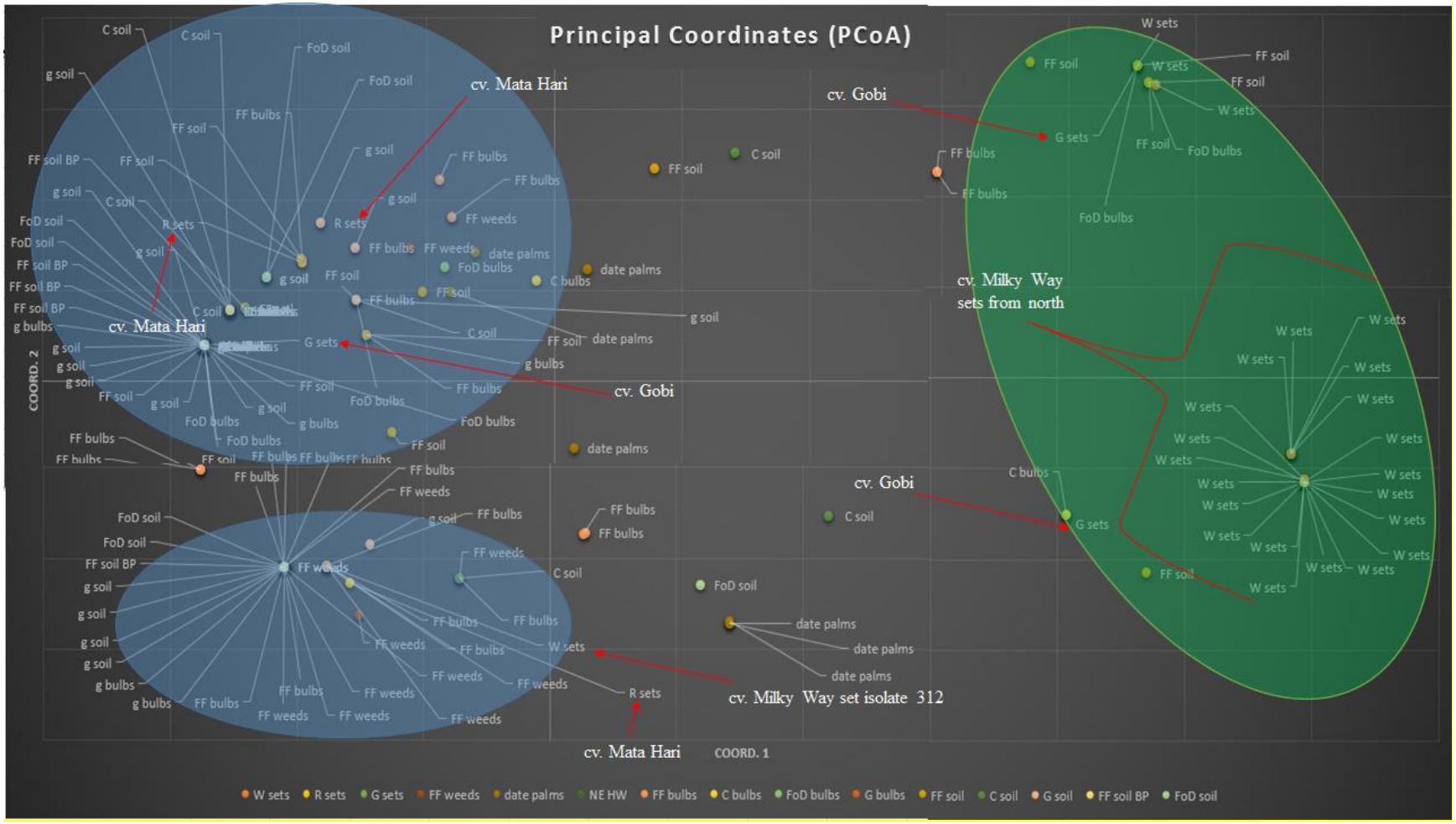

Fig. 14. Principal coordinate analysis of 216 *F. proliferatum* isolates derived from different locations and substrates within Israel.

Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using NTSYS analysis software:

The UPGMA analysis provided a dendrogram of the 216 *F. proliferatum* isolates based on pairwise comparisons of the SSR data. UPMGA defined 49 genotypes (Figure 15), each indicated by a single green line to the left of the isolate name. Multiple isolates shown along a vertical green line are assessed to belong to the same genotype (Figure 15 A). The 49 genotypes fall into 4 main groupings (Figures 15A-15D). The first grouping (Figure 15 A) contains 12 genotypes. Genotype 1 consists of isolates from the Yotvata field soil (collected both before and after the sets were planted), Arava 1 soil, and Grofit soil. Genotype 8 includes the isolates from the salt cedar volunteers inside the cv. Milky Way section of the Yotvata field and some from the cv. Milky Way bulbs from the same field. The second of the four major groupings (Figure 15 B) contains the largest genotype, 13, which includes some isolates from the soils and some from the bulbs of all 4 southern fields. The third major grouping (Figure 15 C) contained all of the isolates from the date palms around the Yotvata field, which comprised 5 distinct genotypes that are very different genetically, based on the pairwise similarity from the 216 isolates. Major grouping 4 contains all of the isolates from the onion sets, collected in northern Israel, and these can be differentiated into three distinct genotypes. The phylogenetic separation of isolates from northern Israel (onion sets) and southern Israel (onion bulbs, weeds, date palms, salt cedars, and production field soils) seen in the dendrogram is consistent with the separation observed with AMOVA, STRUCTURE, BioNumerics, and PCA analyses.

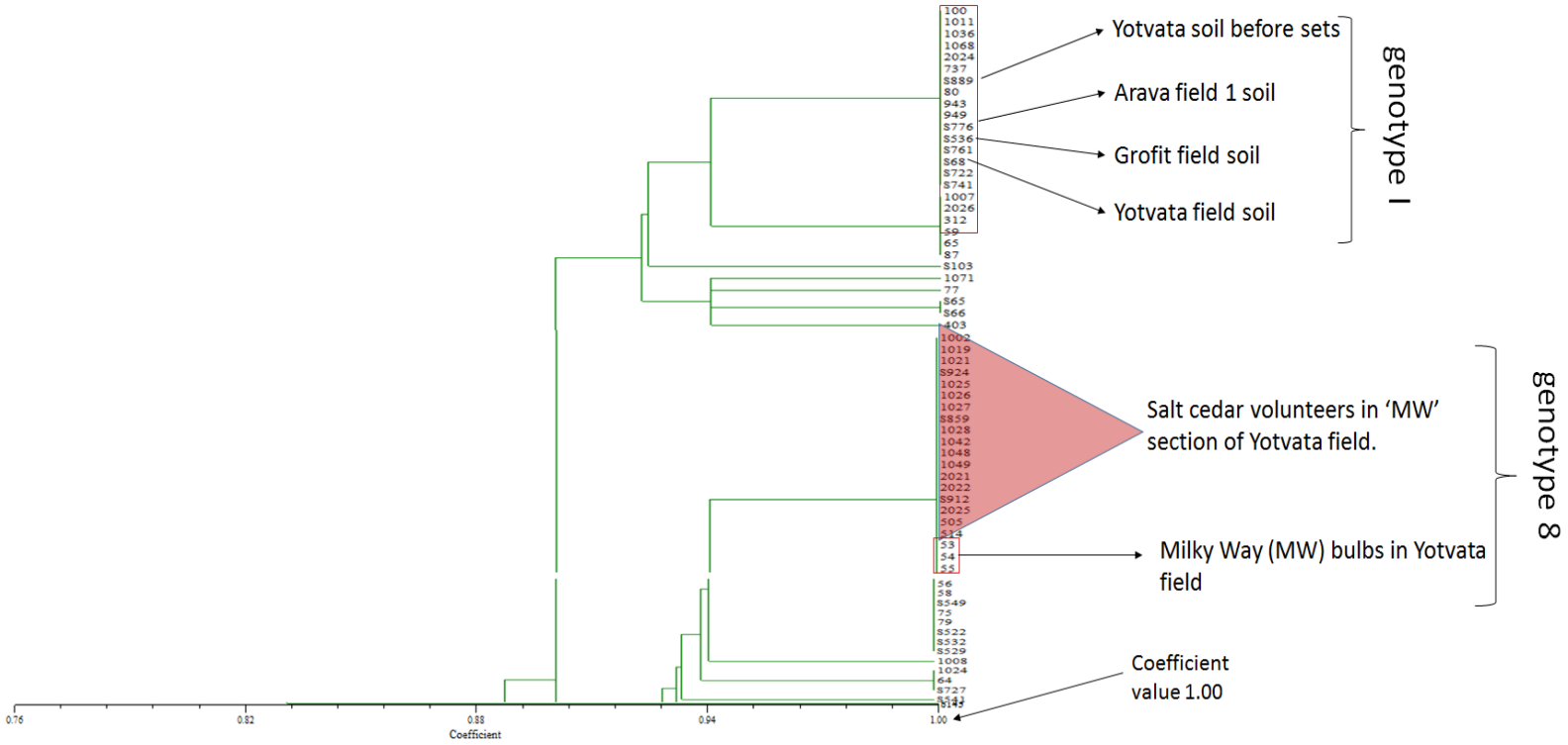

Fig. 15 A. The uppermost portion of the UPGMA dendrogram consisting of isolates belonging to genotypes 1-12. Red boxes and triangle highlight certain isolates within the dendrogram. A coefficient value of 1.00 (bottom of dendrogram) indicates that the isolates are 100% their own genotype.

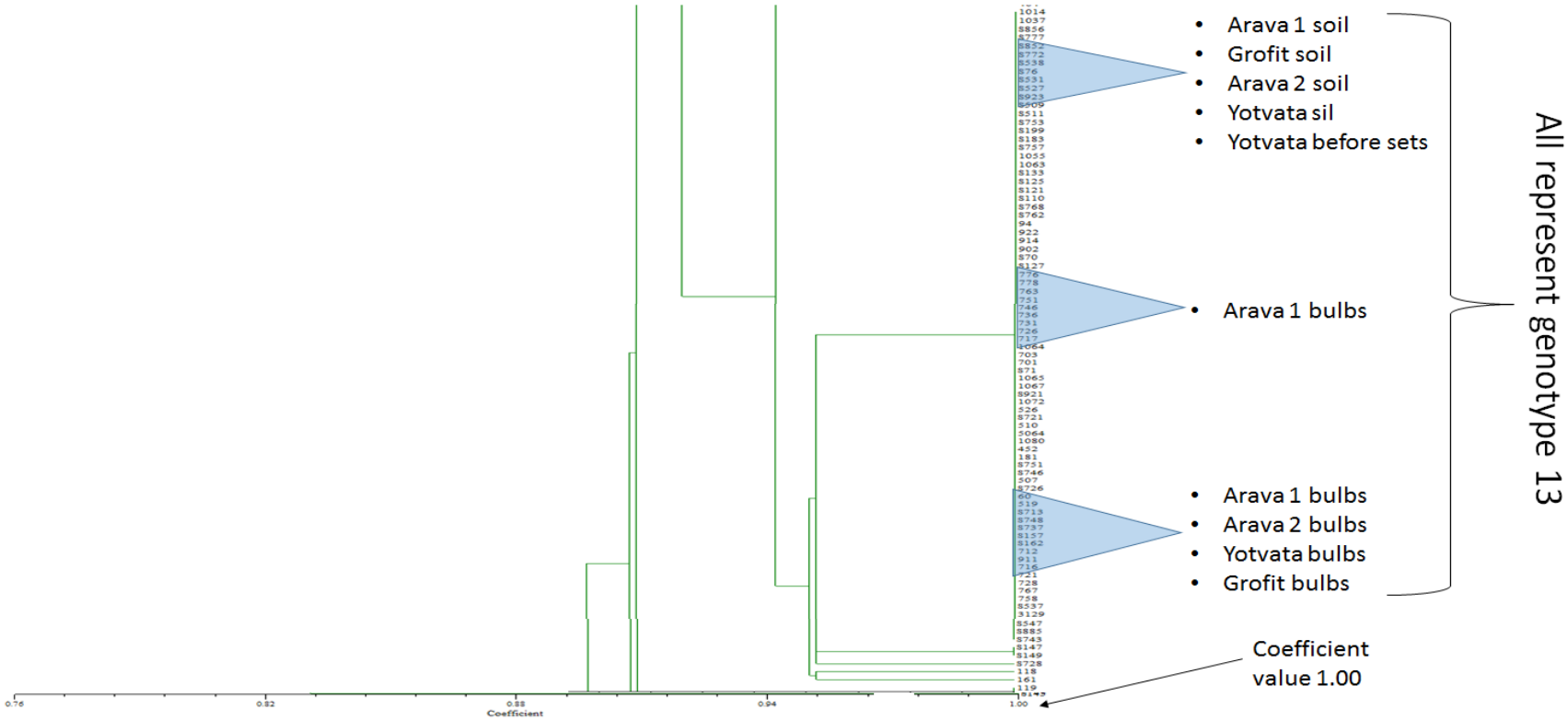

Fig. 15 B. The second major grouping of the UPGMA dendrogram consisting of isolates belonging to genotypes 13-17. Blue triangles highlight certain isolates within the dendrogram. A coefficient value of 1.00 (bottom of dendrogram) indicates that the isolates are 100% their own genotype.

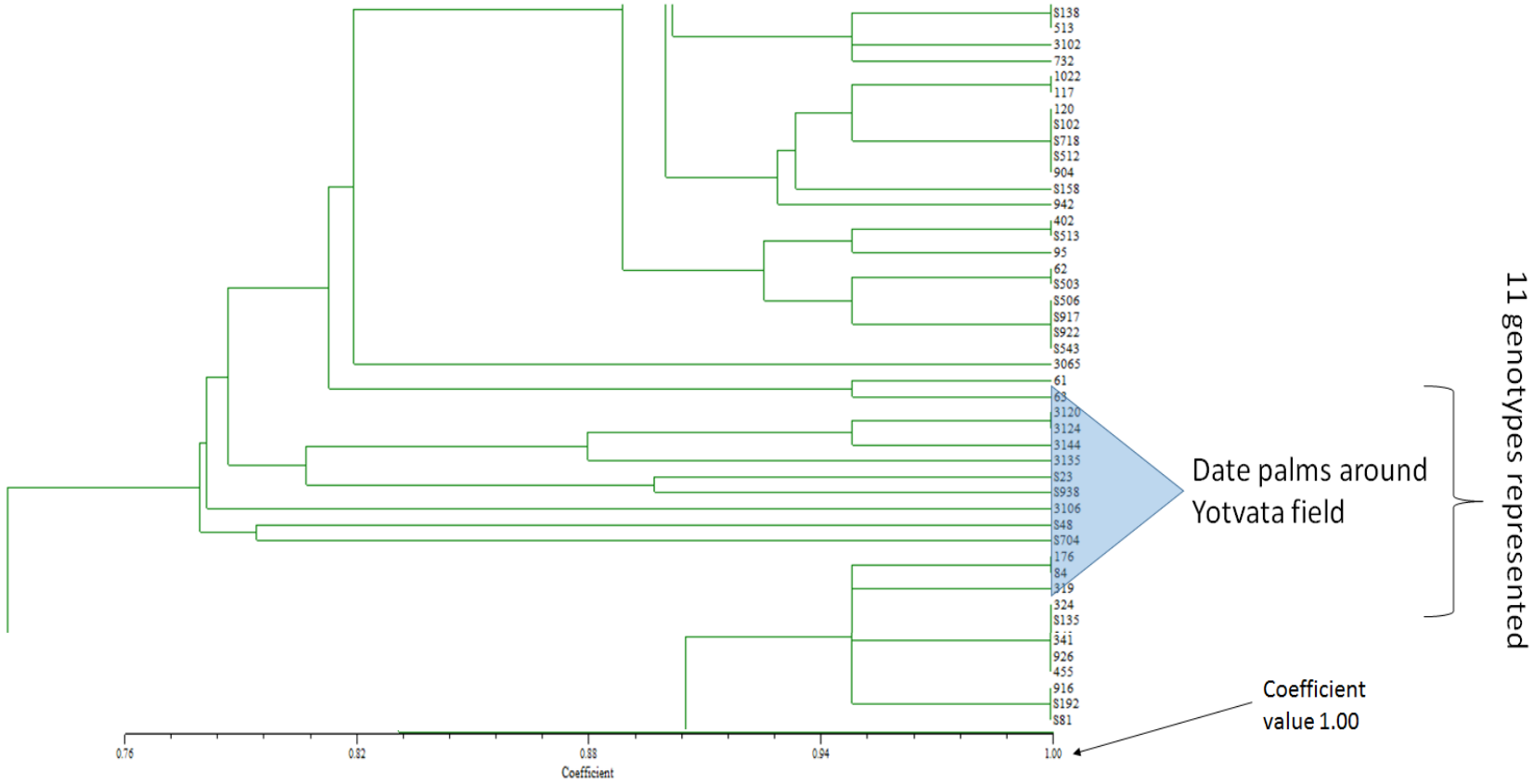

Fig. 15 C. The third major grouping of the UPGMA dendrogram consisting of isolates belonging to genotypes 18-43. Blue triangle highlights date palm isolates. A coefficient value of 1.00 (bottom of dendrogram) indicates that the isolates are 100% their own genotype.

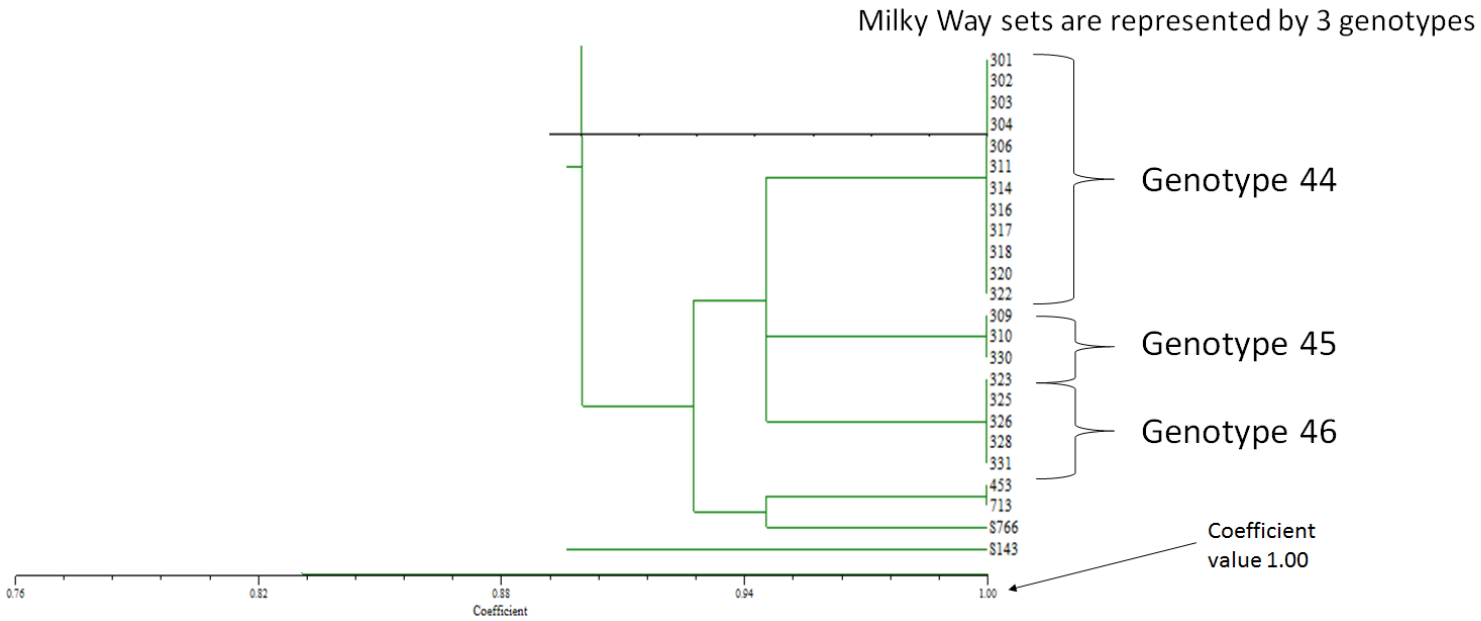

Fig. 15 D. The fourth major grouping of the UPGMA dendrogram consisting primarily of isolates from onion sets belonging to genotypes 44-46. A coefficient value of 1.00 (bottom of dendrogram) indicates that the isolates are 100% their own genotype.

Interpretations/Discussion

The work described in this report represents a unique merger of technologies and strategies of traditional plant pathology, epidemiology and forensic sciences. The recent discovery and rapid severity increases of a new disease, salmon blotch of onions, in Israel served as a highly suitable framework for the field validation of several technologies previously developed and validated in the laboratory. From a plant pathology perspective, we hypothesized that the 2012 salmon blotch outbreak in southern Israel was caused by a strain or strains of *F. proliferatum* present in the onion sets grown in northern Israel and shipped for planting in commercial onion production fields in the south. An alternative hypothesis is that the pathogen was already endemic in the southern onion production areas and, for reasons that might relate to environmental or host factors, emerged as a serious pathogen only in recent years. From a forensic perspective, the hypothesis concept is replaced by goals of determining whether an incident was the result of a criminal action, and if so deemed, identifying the source of a pathogen and its perpetrator for attribution purposes. The first question, whether or not the incident was the result of a crime, was addressed by applying a decision tool designed to assist investigators in making such judgments in an agricultural setting (Rogers et al. 2012; Moncrief et al. 2014). The study reported here was designed to answer the second question: identifying the source of the pathogen. A fungal population biology analysis based on SSR strain typing was used to understand the diversity and relationships among and between populations of *F. proliferatum* found in a variety of host species or other

substrates, locations in Israel, and times of collection. The data collected, analyzed in a variety of ways, provide substantive support for a specific conclusion to that question.

Disease distribution in the field

Disease distribution within a field can offer significant clues about pathogen behavior relating to the site(s) of initial entry into the field: whether the disease began at one focal point or several, whether pathogen entry was facilitated by prevailing winds or by insect vectors, and whether and in what directions within-field spread occurred. If a criminal action is suspected in a forensic investigation, the disease distribution also can suggest whether human-directed dissemination might have occurred. Spatial disease distribution has been studied for wheat stripe rust for epidemics that start at a focal point (Cowger et al. 2005). After artificially inoculating a wheat field with the rust fungus in a 1.5 by 1.5-m focus, the disease spread was monitored upwind and downwind from the focus and there was no significant difference as to if they disease was more severe based on the prevailing winds (Cowger et al. 2005).

In this work, salmon blotch distribution in the four production fields was determined based on which onion bulbs or adjacent soil samples were positive for *F. proliferatum*. In the Yotvata field the high disease incidence in cv. Milky Way made determination of a disease pattern challenging; at that incidence the effect was relatively uniform throughout the plot. Distribution in bulbs of cv. Ada, and in the soil samples collected adjacent to them, was less uniform, perhaps reflecting the lower disease incidence. In the Grofit and Arava I fields, the disease patterns for cv. Milky Way were relatively uniform, but that in

the Arava 2 field was less so. The information gathered about the disease distribution in these fields will be useful for future studies to examine the epidemiology of *F. proliferatum* not only in onions in Israel, but for other crops in other countries too.

F. proliferatum *isolation from plant and soil substrates*

The presence or absence of *F. proliferatum* in a variety of samples, including both living and non-living substrates, and from both northern and southern Israel, was assessed by cultivation attempts using *F. proliferatum* conducive date agar.

Onion seeds

If the outbreak strains of *F. proliferatum* reached the southern Israel onion production areas via the sets grown in the north, then the sets themselves must have become contaminated, either from the onion seeds or from the environment in the set production area. The fact that *F. proliferatum* was never cultured from seeds of the onion cv. Milky Way left over from planting the northern set field and plated onto the surface of date agar suggests that the fungus was not present in or on the seeds and that the seeds were not the source of the *F. proliferatum* strains causing the recent salmon blotch outbreak. It is possible that a physiological effect, such as the presence of a chemical inhibitor in the seeds, could prevent the fungus from growing out of the seeds. The latter possibility could be addressed by attempting *F. proliferatum* isolation from uncontaminated seed samples spiked with cultured fungus.

Onion sets

Of the four onion set cultivars tested, those of cv. Milky Way had the greatest incidence of fungal contamination; of the 50 set samples, 48 yielded *F. proliferatum* isolates. In contrast, the same number of sets from cvs. Ada, Gobi and Mata Hari resulted in only 0, 3 and 4 isolates, respectively. These findings suggest that *F. proliferatum* infestation of the onion sets is cultivar dependent, and that the white cultivar could be more susceptible to the fungus than the yellow and red cultivars. A similar cultivar-associated phenomenon was seen in the percentages of *F. proliferation* contamination in the southern production fields, as noted below.

Onion bulbs

The incidence of *F. proliferatum* in the Yotvata field onion bulbs was variable, with the highest incidence at 84% in the cv. Milky Way bulbs. Fewer or no isolates were cultured from cvs. Mata Hari, Gobi, and Ada. The isolation data for the cv. Milky Way bulbs is consistent with the data from the cv. Milky Way set isolations. Interestingly, no isolates were cultured from cv. Ada sets, but 22 isolates were cultured from Ada bulbs, suggesting that the *F. proliferatum* isolates from bulbs were likely infested with the isolates after being planted in the Yotvata field soil. Isolates were cultured at low incidence from the cv. Gobi and cv. Mata Hari bulbs. The percentages of *F. proliferatum* isolates cultured from cv. Milky Way bulbs grown in the other three fields in southern Israel varied; 60% in the Grofit field, 80% in the Arava 1 field and 45% in the Arava 2 field.

Soil

In the Yotvata field the soil samples collected from the cv. Milky Way plot had the highest percentage (84%) of isolates cultured, were. Overall, the numbers of isolates from the Yotvata field soil were similar to those collected from the bulbs from that field. This finding is consistent with the hypothesis that the soil was the source of the fungus strains causing the recent salmon blotch outbreak, or with the alternate hypothesis that fungus present in the bulbs contaminates the soil in its immediate vicinity. Neither hypothesis can be tested using the isolation data alone, since it does not reveal whether the *F. proliferatum* isolates from the bulbs match those from the soil.

Interestingly, *F. proliferatum* was cultured from soil samples collected from the Yotvata field before any sets were planted that year. If the latter isolates are similar to those collected during the 2012 outbreak, then the soil may be the source of the strains causing salmon blotch in that field. The results are consistent with the data that the two populations of isolates are similar to each other based on the phylogenetic analyses.

Due to flooding, we were unable to sample soil from the northern set production field, but soil samples collected the previous year from that field yielded no *F. proliferatum* isolates.

Weeds within Yotvata field

Weeds present within the cv. 'Milky Way' area of the Yotvata field included volunteers of salt cedar and date palm, both of which are known hosts of *F. proliferatum* in Israel (Gamliel, personal communication). The recovery of isolates from weeds within the field suggests either that they can be a source of the fungus, or that they acquired the fungus

from the soil or from the infected onion crop. However, as stated above, the phylogenetic relationships among isolates from these weed species and the onions must be determined. Our results are consistent with the data that the populations from the weeds inside of the cv. 'Milky Way' are similar to the soil and the onion bulbs in the Yotvata field.

Windbreaks, date palm plantation, highway perimeters

The vegetation closest to the Yotvata field were salt cedar trees planted as windbreaks. *F. proliferatum* was never cultured from any of these trees, suggesting that the fungus was not present in them, or that a compound within the trees suppressed fungal growth. Isolates were, however, cultured from date palm trees in two plantations, both at least a decade old, located east and south of the Yotvata onion field. Only one isolate was cultured from the vegetation near the Yotvata highway, specifically from the plant, *Acacia tortilis* (Forssk.) Hayne. It would not be surprising to find that the fungus is rare in that location, since this part of southern Israel is arid, irrigation used in agricultural production does not reach the roadsides, and vegetation along the highway is sparse

SSR loci of F. proliferatum *isolates*

While disease incidences and in-field pattern data, such as those described above, provide important insights into the history and evolution of a particular disease outbreak, conclusions about pathogen origins, host ranges and movements cannot be made without understanding the relationships among isolates from each of these populations. In this study, SSR analyses were used to determine relationships among *F. proliferatum* isolates from different populations, locations, hosts, and times of collection.

Six previously described SSR loci (Moncrief, 2014, Chapter 3) were amplified from 216 out of 309 *F. proliferatum* isolates tested. The failure of the SSR primers to amplify the other 93 isolates could be due to the fact that the latter lack the repeat motifs for which the primers were designed, or that some of the isolates were mis-identified as *F. proliferatum.* The *F. proliferatum* isolates used in this study were identified only using morphological characteristics. Confirmation of fungal identity could be done by testing putatively identified *F. proliferatum* using species specific primers to confirm the morphological data.

AMOVA analysis of F. proliferatum *populations*

AMOVA analyses demonstrated that the *F. proliferatum* isolates from the onion sets from northern Israel belong to a different population than all isolates collected in the south, based on the PhiPT value, 0.655. The set isolates were assessed to be a different population than that of bulbs grown in the south, suggesting that the sets are unlikely to be the source of the fungus (PhiPT = 0.7). If the sets were the source of *F. proliferatum*, then a PhiPT value <0.1 would be expected. In contrast, isolates from the Yotvata soil at the time of bulb maturity vs. those collected before the sets were planted in the field, show a PhiPT value <0.05, which indicates that these *F. proliferatum* isolates are clonal. *F. proliferatum* is known to survive in fields for several years (Cotton et al. 1998) and it is possible that the Yotvata field soil was the source of the fungus responsible for the current outbreak of salmon blotch. This interpretation is consistent with the data

comparing the PhiPT values between the soils of the Yotvata field and the other three fields (PhiPT <0.05).

Weeds have been reported to be hosts of *F. proliferatum* (Postic et al. 2012) and we obtained isolates from several different weed species, including volunteer salt cedar seedlings and date palm seedlings growing within the white onion bulb plots in the Yotvata field. The AMOVA analysis between the two populations revealed a PhiPT value of 0.185, which indicates that they are different populations, albeit of relatively low genetic diversity. Furthermore, isolates from white onion bulbs comprised a different population than those from date palm seedlings, based on a PhiPT value of 0.254. On the other hand, the PhiPT value comparing the white onion bulbs to the salt cedar volunteers is 0, consistent with our interpretation that the isolates from these two populations are clonal. The data suggest that salt cedar can be an alternative host to salmon blotch strains of *F. proliferatum* in Israel. Interestingly, the isolates from the 'Milky Way' portion of the soil are moderately different, genetically, from the isolates from the 'Ada' portion of the field, even though the two cultivars are separated by only one furrow. This finding may reflect multiple populations of the fungus in the soil, or uneven distribution of the populations within the field. Furthermore, our failure to recover *F. proliferatum* from the 'Ada' sets suggests that the isolates cultured from the 'Ada' bulbs infected the bulbs after their arrival in southern Israel.

BioNumerics minimum spanning tree analysis

The BioNumerics software suite is used commonly to strain-type bacterial species involved in foodborne disease outbreaks (Swaminathan et al. 2001). Using this analysis, most of the *F. proliferatum* isolates from the sets from the north are separate from southern *F. proliferatum* isolates (clusters A and B). These results are consistent with those of the AMOVA analysis. The 21 groupings (circles) suggest 21 genotypes within this species. The majority of the weed isolates, including those from the salt cedars inside the Yotvata field (cluster A), are of the same genotype as isolates from the Yotvata field bulbs. The *F. proliferatum* isolates from date palms form a separate cluster (B), indicating that they are a separate population from that of the Yotvata field weeds, and mostly separate from the onion bulbs grown in the south. The majority of the soil isolates group in cluster A, but a few are distributed among the four clusters. These data suggest that there could be movement of the fungus in the south.

STRUCTURE analysis

The STUCTURE analysis grouped the 216 isolates into two populations, similar to the outcomes of the AMOVA and BioNumerics analyses, in that the onion sets (grown in the north) are separated from the southern isolates. This analysis also provides evidence that some isolates from the north could be hybridizing with some isolates from the south. This could be possible if an isolate was moved from the north on an onion set, planted in the south and then stayed in that field for several years among the *F. proliferatum* isolates already present in the field. Over time, an exchange of genetic material can result by the

fusing of hyphae. The finding indicates the possibility of the fungus being disseminated across southern Israel by prevailing winds. One isolate, 312, from the cv. Milky Way onion sets showed >99% similarity to the isolates from the south. This finding could indicate that the onion set from which isolate 312 was isolated was contaminated with the soil from the Yotvata field, not planted, and brought back to the lab for storage. It could also mean that there is great diversity of *F. proliferatum* throughout Israel brought about the movement of the fungus from the north to the south or vice versa.

GeneAlex principal component analysis

The PCA is consistent with the AMOVA and STRUCTURE analyses in that the white sets from the north form a group that is separate from the isolates collected from southern Israel. The date palm isolates form two separate clusters within the larger population of isolates from the south. The date palm plantation west of the Yotvata field has been established for over 10 years, while the date palm isolates from the oldest plantation (20 years old), to the south of the Yotvata field group, are distinguishable from the other southern isolates. It is possible based, on this data, that multiple genotypes of *F. proliferatum* have been introduced to the south over the years. The red and yellow set isolates, which are scattered within the southern population, group separately from with the white set isolate population, a finding consistent with the data from the STRUCTURE analysis. These isolate groupings also are similar to those observed in the BioNumerics minimum spanning tree analysis.

UPGMA analysis

One of the most notable results of the UPGMA dendrogram is that the *F. proliferatum* isolates from the white onion sets form a clade separate from that of the other set isolates and separate from that of the isolates collected from the south. These data are consistent with the previous analyses and with a conclusion that the sets are unlikely to be the source of the outbreak pathogen in Israel. The southern soil isolates are distributed throughout the entire dendrogram; one small clade containing one isolate from each field, indicating that these isolates could be clonal in nature. This finding suggests that the fungus can be spread, by wind or another means, to nearby fields. As seen with the other analyses, the date palm isolates form a unique clade unrelated to the isolates responsible for the salmon blotch outbreak in Israel. The salt cedar isolates from the Yotvata field grouped into the same clade as that of the white bulbs, indicating that these two groups of isolates are clonal. This conclusion, which is supported by the AMOVA analysis, suggests that salt cedar can be a host of the salmon blotch strain of the fungus and could have been a source for the recent salmon blotch outbreak. The fact that we were unable to isolate the fungus from the mature salt cedar trees around the Yotvata field is unexplained. Perhaps we sampled a part of the tree that was not colonized and missed the fungus all together, or perhaps a physiological inhibitor in the mature trees prevented the fungus from colonizing the mature trees.. Our interpretation that the soil in the south could be a source of the fungus is consistent with the data because the isolates collected from the Yotvata soil, before any sets were planted, fall into the same clade as those collected during the early investigation of the 2012 outbreak.

Overall, the results of the phylogenetic analyses are consistent, all pointing to a conclusion that the onion sets are unlikely to be the source of the salmon blotch outbreak, based on the fact that they group separately from the rest of the isolates from southern Israel. Further, the *F. proliferatum* isolates from date palm plantations, which have been in the Yotvata area for over 20 years, are genetically different from the southern isolates. The *F. proliferatum* isolates from all four field sites are similar to one another and the isolates cultured from the soil in the Yotvata field, before the sets were planted, match those collected during our investigation. *F. proliferatum* has been found in the northern set fields (Gamliel, personal communication), but in this study we were unable to collect samples from that area *F. proliferatum* isolates from volunteer salt cedar plants within the cv. Milky Way section of the Yotvata field match the pathogen isolates from the soil and the bulbs collected in that section, based on the phylogenetic analyses. It is possible that *F. proliferatum* is endemic in various plants and soils in southern Israel.

SSRs are powerful molecular markers that are useful for identification, phylogenetic analysis and traceback of a fungus and are useful for forensic analysis applications. Their discriminatory power was demonstrated by the capacity to differentiate isolates from northern Israel from those in southern Israel. Based on the SSR analyses, we conclude that the onion sets are not the source of the *F. proliferatum* causing the salmon blotch outbreak.

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CHAPTER VI

The application of a decision tool to investigate whether or not an outbreak of salmon blotch of onions in Israel is a result of a natural occurrence or a biocrime

Abstract

Agriculture is a vulnerable to plant pathogens introduced naturally or by harmful intent. Law enforcement personnel conducting a forensic investigation may not be familiar with the agricultural setting. Previously, a self-guiding decision tool, modeled for the plant pathogen *Wheat streak mosaic virus*, was designed to help such investigators assess the likelihood that the outbreak was intentionally caused. In the study reported here, the tool was adapted for the plant pathogenic fungus *Fusarium proliferatum* and its efficacy was assessed by applying it to an investigation the source of the fungus causing an outbreak of salmon blotch of onion in southern Israel.

Introduction

Plant pathogen forensics, an emerging discipline that blends the science of microbial forensics with the concepts of plant pathology, enhances U.S. agricultural biosecurity (Fletcher et al. 2006). Prior to a forensic investigation, it is essential to determine if a crime has been committed. This determination can be particularly challenging in forensic plant pathology due to the lag time that occurs between pathogen introductions and disease development. Sometimes it can take several weeks for disease symptoms to manifest, which makes it hard to determine if the disease is a result of natural factors or intentionally incited. Pathogens are often imported inadvertently and disseminated to previously unaffected areas.

Biocrimes and or bioterrorism are the threat or actual use of microorganisms, toxins, pests, or prions to commit criminal or terrorist acts (Breeze et al. 2005). In the case of plant pathogen forensics, a biocrime could be a result of a grower sabotaging the field of a competing grower to eliminate competition or due to a personal dispute. An example of a bioterrorism event is a political group releasing a pathogen to weaken a country's agricultural sector for political gain. As yet, no acts of agricultural bioterrorism against the U.S. have been confirmed, but we should be prepared to deal with such matters should they arise. A tool designed to assist investigators in assessing whether a disease outbreak was due to natural events or to human involvement could facilitate decision-making and shorten the time for a response to a biocrime.

There is precedent for the use of a decision tool to assess whether an outbreak of a disease is intentional or due to natural causes. After an epidemic of the human disease

tularemia, caused by the pathogen *Francisella tularensis*, occurred in Kosovo from 1999-2000, Grunow and Finke (2002) developed such a decision tool and used it retrospectively to assess the likelihood that the outbreak was intentional. By rating a series of characteristics related to the disease, the pathogen, and elements such as the political and social environment, and then applying appropriate weighting factors, they ruled out the possibility that the tularemia epidemic was a result of biocrime and concluded that the likely source of the pathogen was rodents in Kosovo (Grunow and Finke, 2002). A decision tool, modified from that of Grunow and Finke (2002), was developed by Rogers (2011) for the plant pathogen, *Wheat streak mosaic virus* (WSMV) in Oklahoma wheat fields. The decision criteria used in this tool, which was designated the Crop Bioagent Introduction Assessment Tool (CBIIAT), were relevant to the pathogen host range, environmental conditions, epidemiology, dissemination, and other disease-related and situational elements. The tool was validated in one growing season by the investigator, and in a second season by a group of law enforcement personnel and extension agents, who used it to assess intent at two wheat fields, one that was intentionally inoculated with the virus and another that had a natural infestation of the virus.

The aims of this study were to (1) adapt the decision tool for a different pathosystem, the fungus *F. proliferatum* and the disease salmon blotch of onion (Isack et al. 2014), and (2) to assess its effectiveness in an actual field setting.

Salmon blotch disease in Israel

In the summer of 2005-06, salmon pink blotches were observed on the surfaces of some white onions in commercial fields located in Yotvata, Israel. The discoloration continued in the inner 2nd, 3rd, 4th, 5th, and 6th layers and some onions eventually rotted (D. Gilette, personal communication). Whether these symptoms represented a primary or secondary infection was unclear. A fungus was consistently isolated from symptomatic bulbs and onions re-infected with that fungus developed salmon-colored blotches, fulfilling Koch's Postulates (Gamliel, personal communication). The fungus was identified by PCR as *Fusarium proliferatum* (Gamliel, personal communication).

F. proliferatum produces a mycotoxin, fumonisin, which poses health risks to humans and animals if ingested. In Israel, the highest levels of mycotoxin are produced in white onion cultivars and there is less toxin in yellow and red cultivars (Gamliel, personal communication).

Onion production is Israel

Onion seeds, which are either imported or produced within Israel (Gamliel, personal communication), are planted in in northern Israel around the third week of January. Once the seeds produce sets (small bulbs) around mid-February they are harvested and stored in sheds until they are sold to production farms in southern Israel. The sets are planted directly in the soil toward the end of August or early September and grow into mature bulbs, which are harvested in January or February before being sent to the local packing houses and sold. A large commercial onion field, designated the Yotvata field served as the primary site for the decision tool assessment. It contained

rows planted with white (cv. Milky Way), yellow (cvs. Ada and Gobi), and red (cv. Mata Hari) onions. Three other locations, the 'Grofit' field (in the nearby kibbutz town of Grofit) and two research fields, Arava field 1 and Arava field 2; both owned by the Arava Research and Development Experiment Station, Arava, Israel) were also planted with white onion (cv. Milky Way) (Figure 1).

The *F. proliferatum* – onion pathosystem makes a good model system for several reasons. First, *F. proliferatum* infects a wide range of hosts and is easily isolated. Second, the production mycotoxins makes the disease a potential biosecurity issue. Finally, salmon blotch is relatively new to Israel, having been first seen in the early 2000s before being identified in 2008. The 2013 outbreak served as an opportunity to apply the decision tool to an authentic incident.

Figure 1. Overhead view of the Yotvata region and the four field sites.

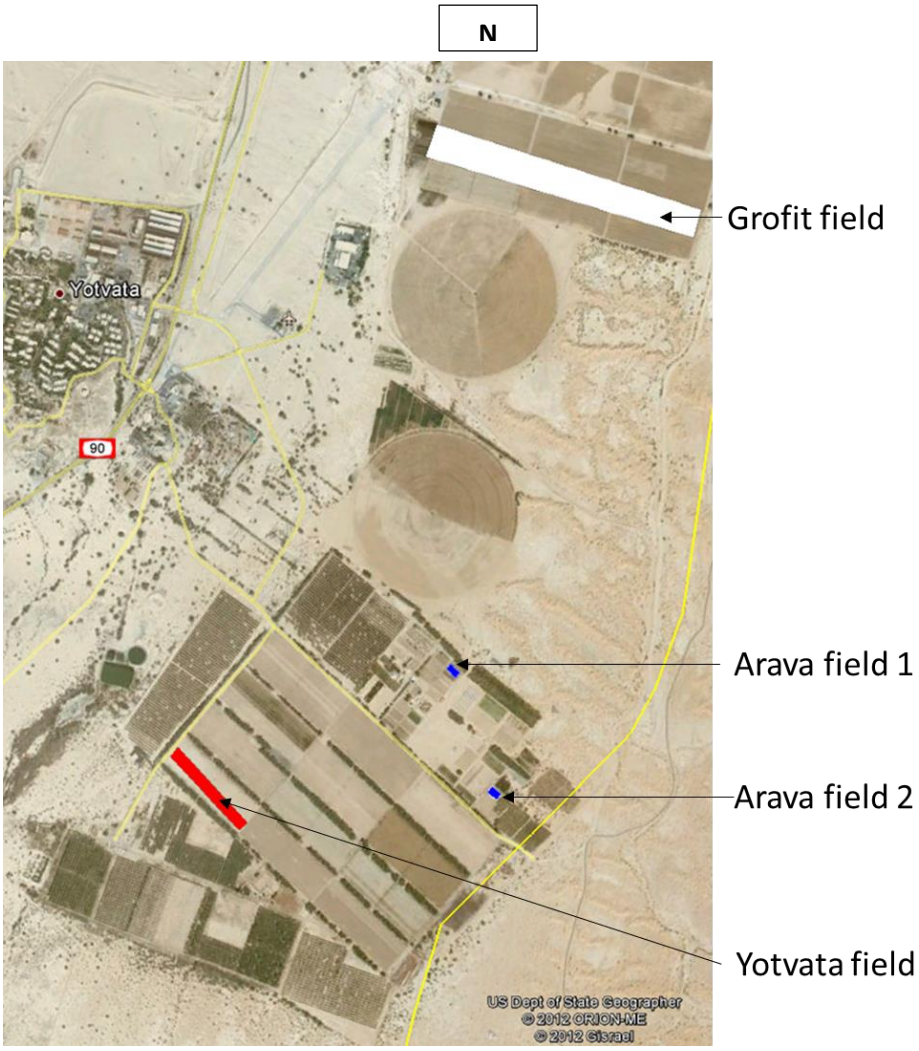
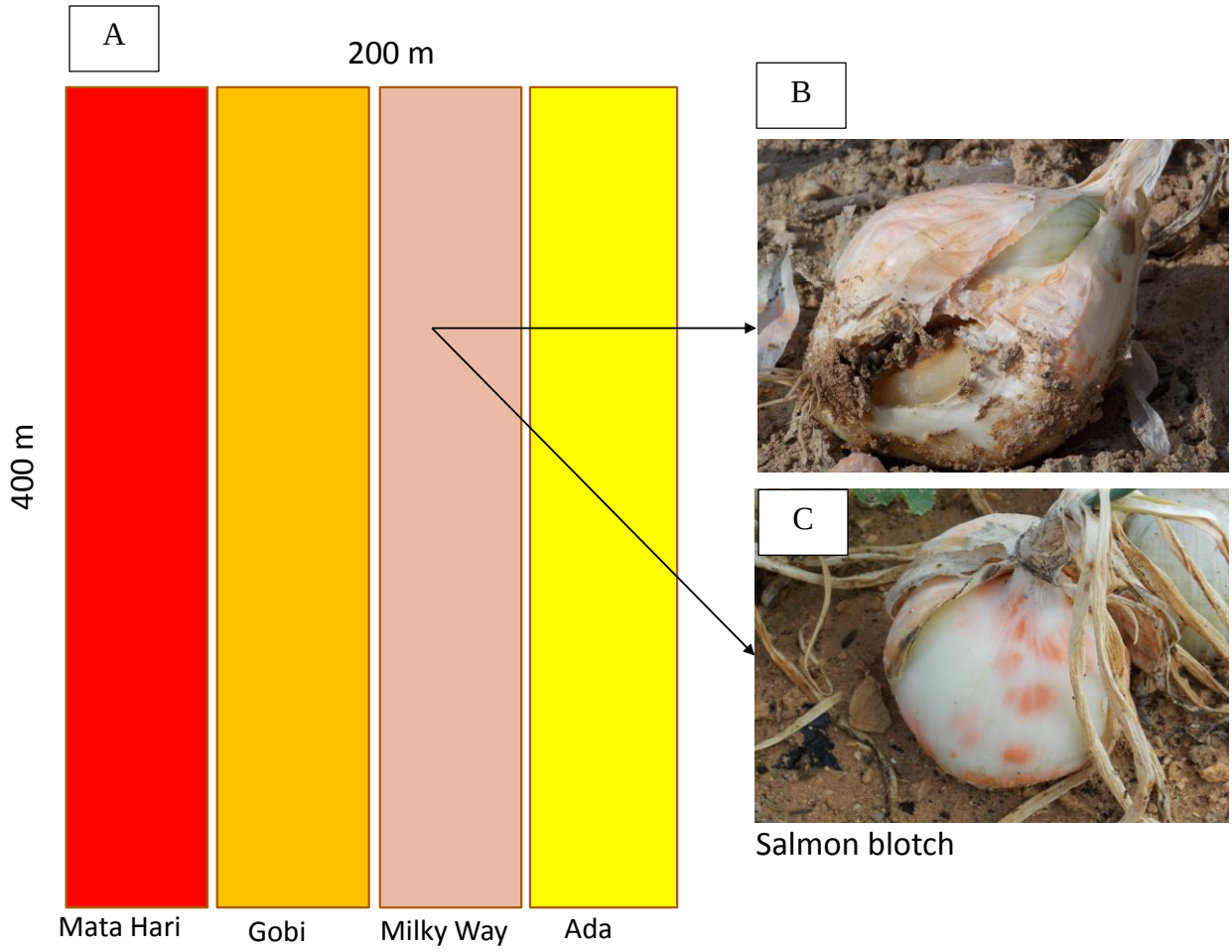


Figure 2. Schematic of the Yotvata field illustrating (A) the positions of the red, yellow and white cultivars, (B) bulbs rot at late stages of disease development, and (C) the white/pink blotches that are signs of the pathogen.



Materials and Methods

A commercial field owned and farmed by, and located on, the Arava Research and Development Experiment Station by a local grower in Yotvata, Israel, was used as the primary site for assessing the decision tool. Several crops were rotated in the field over the previous decade but in 2012 onion sets (young bulbs) of four cultivars, two yellow, Ada (A) and Gobi (G), one white, Milky Way (MW), and one red, Mata Hari

(MH) were planted. The field, 200m x 400m in size, is surrounded by windbreaks of mature salt cedar trees and flanked on two side by date palm plantations. Inside the field, in addition to onions, are variety of weeds and volunteer plants. The field is drip-irrigated with water from a local well. The water and the sandy soil have a high salinity content of .5 µM and ~3.0 µM respectively (Gamliel, personal communication).

As the bulbs reached maturity in November of 2012, salmon blotch symptoms were observed on the outer scales on almost all of the MW cultivar bulbs. Disease symptoms were not visible on bulbs of the other three onion cultivars, even though only one furrow separated each pair of cultivars. The grower harvested and sold the yellow and red cultivar onions, but he did not market the MW bulbs due to the possibility that they contained mycotoxins produced by *F. proliferatum*. In 2012, the salmon blotch incidence in the Yotvata field was the highest ever seen in the area since the disease was identified in the area in 2008.

Selection of decision tool criteria

A decision tool for assessing the possibility of human involvement being responsible for this outbreak of salmon blotch of onion consists of eleven criteria related to the pathogen-host disease cycle. They relate to 1) geographical distribution, 2) spatial distribution, 3) weather, 4) temporal issues, 5) field history and cultural practices, 6) crop rotation, 7) human activity, 8) physical evidence, 9) motive, 10) surrounding areas, and 11) pathogen features (Table 1).

Criteria and their weighting factors

For each criterion, a weighting factor of '1', '2', or '3' was assigned depending on the degree to which that criterion impacted the assessment (Table 3).

I. Geographical distribution of *F. proliferatum* in Israel (weighting factor of 3):

F. proliferatum present in local soils and/or vegetation could have served as an inoculum source for the onion bulbs, inciting the disease observed in southern Israel. Alternatively, *F. proliferatum* that was already present in the onion sets, produced in northern Israel and shipped to be planted in the south, could be responsible. Sampling the sets, as well as the soils in the set fields in the north could help determine if the onion sets were the source of the fungus. Salmon blotch has not been reported previously in the set production areas (Gamliel, personal communication) and we were not able to visit those sites during our investigation due to heavy flooding. Attempts to recover the fungus from soil samples collected from the set fields a year before our investigation were unsuccessful.

II. Spatial distribution of *F. proliferatum* in the Yotvata field (weighting factor of 2):

The 'normal' infection pattern of *F. proliferatum* of onions is not known. Growers in southern Israel recall that in previous salmon blotch outbreaks disease symptoms were uniform throughout plantings of white onions (D. Gilette, personal communication). The symptoms are less visible on yellow and red onions, so it is difficult to assess how the disease is spread through those cultivars. If the disease pattern is patchy, it could mean that there were multiple infection points, while a concentration of

disease along one field edge could be the result of windborne spores arriving from the direction of the prevailing winds.

III. Weather (weighting factor of 3):

In general, for a plant disease to be incited, symptoms will appear only if the environmental conditions are favorable. The optimal temperature for vegetative growth of *F. proliferatum* is 28°C (Leslie and Summerell, 2006). Microconidia germination is optimal at 30°C, regardless of humidity levels, but there is high variation among different isolates in the lag time until spore germination (Popovski and Celar, 2012). The occurrence of a plant disease during a period in which the weather is not conducive raises the question whether outside influences could be involved.

IV. Temporal factors for *F. proliferatum* (weighting factor of 1 and 3):

This criterion was divided into two questions, 1) Is this the usual time of year for a salmon blotch outbreak? ; and 2) Is this the usual severity of symptoms for the time of year? Salmon blotch appears late in the growing season, when the bulbs near maturity (Gamliel, personal communication). Disease symptoms (Figure 2, seen on white onion cultivars) and the severity and incidence of the disease can vary among onion fields. For example, if all of the onion bulbs in a field showed salmon blotch but only 20% of the bulbs were rotted, then the disease incidence would be 100% but the severity could be considered low. Alternatively, a field could have 30 % of the bulbs showing symptoms with all of them are rotted, which would indicate a low disease incidence but a high severity.

V. Field history and cultural practices (weighting factor of 1):

Field history includes information of previous incidents to serve as a baseline for comparison. The occurrence of a new disease or its appearance in a new location will be indicators of the need for further investigation. Cultural practices such as chemical applications, soil solarization, field tilling, among others, that can influence the outcome of a disease are also of interest.

VI. Crop rotation (weighting factor of 1):

F. *proliferatum* has a wide host range and can survive in plant debris from one growing season to the next (Cotton and Munkvold, 1998). In the Yotvata field, the grower rotates between potatoes, sweet corn and onions, and sometimes leaves the land fallow (D. Gilette, personal communication). In 2007, he observed salmon colored blotches on the white onions. *F. proliferatum* survives in fields for several years, even though it does not produce resting spores (Leslie and Summerell, 2006), and could be a source of the fungus in following years. It has been reported to infect maize (Alizadeh et al. 2010) but there are no reports of the fungus being isolated from potato.

VII. Human activity (weighting factor of 3):

Farm operations usually have a lot of human activity in and around the field(s). Vehicles and farm equipment may enter the fields and even aircraft, such as crop dusters, may visit the fields. However, unusual types of human activity within or around a field may be suspicious. Examples include personnel entering unauthorized areas, spraying in a field when it is not ordered or during unusual hours of the day, and unauthorized crop dusting. Although growers are watchful, it is impossible to monitor every operation 24 hours a day.

VIII. Physical evidence (weighting factor of 3):

Evidence found and collected at a crime scene can be used to link a suspect to the crime. Types of physical evidence that could be associated with the intentional release of a plant pathogen, such as *F. proliferatum*, would include pieces of laboratory equipment or supplies, sprayers or other delivery systems articles of clothing, and unusual tire tracks in the field.

IX. Motive (weighting factor of 3 for first part, and 3 for second part):

Investigators will look for a motive that would give anyone a reason to commit a biocrime. This criterion was divided into two segments, 1) no motivation to harm the grower, and 2) no evidence of a national attack. Motivation to harm a grower or his field(s) can be personal, such as a grudge between an employee, family member, or a neighboring grower. An employee who was recently fired might lash out at the grower or sabotage the field. Disagreements among the grower's family could lead to sabotage of the crop. Motivation to harm a grower at the local level could be triggered by jealousy if one grower is out-competing the rest. The second part of this criterion relates to the possibility of state sponsored activities. Political, religious, or social tensions among different factions within a country or between countries could be motives for international nefarious actions.

X. Surrounding areas around the Yotvata field (weighting factor of 1):

F. proliferatum has been isolated previously from date palms, a variety of weed species, and salt cedar in southern Israel (Gamliel, personal communication). Two perimeters closest to the Yotvata field are (1) the salt cedar windbreaks and (2) date palm

plantations (Figure 3). Further from the field, (3) vegetation near a main highway that runs from northern to southern Israel (Figure 4). If *F. proliferatum* is found in these surrounding areas it is possible that they could be the source of the fungus causing salmon blotch in the Yotvata field. The highway perimeter was chosen as the collection site farthest from the Yotvata field.

Figure 3. Overhead view of the Yotvata field and the surrounding perimeters, salt cedars (1) and the date palm plantations (2).

Figure 4. Overhead view of the collection site farthest from the Yotvata field.

XI. Pathogen characteristics (weighting factor of 2):

Characteristics of *F. proliferatum* can be useful for the decision tool. Morphological features such as mycelium color and spore shape can be used to distinguish different species of *Fusarium*. However, *F. proliferatum*'s production of various pigments in its mycelium can lead to misidentification. Molecular characteristics such as DNA fingerprints among isolates of *F. proliferatum* from onion sets, soil, plants, and bulbs can inform assessments of possible sources of the fungus producing the salmon blotch symptoms in southern Israel. A close match between the DNA fingerprints from the isolates from the infected onion bulbs and those of another group of isolates (for example, the isolates from the onion sets) could implicate the latter as the source of the outbreak fungus.

Assigning assessment values

During the decision tool assessment process, the user is asked to input an assessment value for each criterion statement based on observations in the field, in the lab, or from interviews with victims and other relevant individuals (Table 2). A value of '1' indicates that the statement is in full agreement with the field situation, '2' indicates that the statement is partially valid and/or partially invalid, based on the field situation, and '3' indicates that the statement does not match the field situation at all.

How the decision tool was used in this study

To adapt the decision tool for the Yotvata field assessment, relative literature pertaining to the host, the pathogen and the disease, as well as to the farm production system, was collected. Weather data sources such as the National Climatic Data Center (http://www.ncdc.noaa.gov/) were searched for average temperatures near Yotvata during the time the onion sets were planted. Parts of the decision tool requires the investigator(s) to interview persons of interest, such as growers, employees, extension personnel, professors, and others of interest. In this study, the grower who rents the Yotvata field, the extension specialist and head of vegetables research, and a plant pathology researcher from the Volcani Institute who conducts experiments at the experiment station were interviewed. A few of the tool criteria can be answered only after sample collection and lab analyses are completed. A team of researchers, including the assessor, collected soil, plant, and onion samples from the Yotvata field and the surrounding areas. The *F. proliferatum* isolates were identified morphologically in Israel and then shipped to Oklahoma State University (Stillwater, OK) for molecular analysis.

Since law enforcement personnel investigating a suspicious disease outbreak would have to make an initial assessment, based only on field observations and witness interviews, about whether anything was unusual at the field, a further more inclusive forensic investigation would be warranted. The decision tool of the disease in the Yotvata field was performed twice in this study, once in the initial stages of the investigation (criteria I-IX) and a second time after the incorporation of the lab results (criteria I-XI).

Simple sequence repeat (SSR) analyses

SSR markers have been identified in some *Fusarium* species, such as *F. verticillioides,* which is closely related to *F. proliferatum* based on phylogeny (Leyva-Madrigal et al. 2014). SSR markers have been identified in *F. proliferatum* (Moncrief et al. 2014, Chapter III of thesis) and validated on 10 isolates of the fungus from Germany, Israel, and North America from onion, asparagus, and maize. These SSR primers were used in this study to characterize populations of the fungus from the plant and soil materials collected during the Yotvata field investigation.

Results

Early assessment of the forensic field

Criterion I: Geographical distribution of *F. proliferatum* in Israel:

F. proliferatum was recovered from plant and soil samples collected in southern Israel, in and around the onion field, from adjacent windbreaks, date palms, soils, and weeds. *F. proliferatum* was not recovered from the set field soils in northern Israel; we were not able to visit the set fields due to heavy flooding, and set field soil collected the

previous year yielded no *F. proliferatum* cultures. The SSR analysis suggests that the isolates from the 'Milky Way' cultivar sets are closely related to one another, but that they differ genetically from isolates recovered from the infected onion bulbs from southern Israel. Because *F. proliferatum* had been reported in southern Israel in the past, and was detected in this study in southern Israel vegetation and soils outside of the Yotvata field, an assessment value of'1' was assigned to this criterion.

Criterion II: Spatial distribution of *F. proliferatum* in the Yotvata field:

Observations of disease incidence in the field and the distribution of bulbs from which *F. proliferatum* was isolated were contributing factors in the assessment. Two yellow, one red and one white onion cultivar were planted in the Yotvata field. The disease incidence in the white onions was 100%: No salmon blotch symptoms were visible on the outer scales of the white (cv. Milky Way) onion bulbs. The fungus incidence in the two yellow onion cultivars, Ada and Gobi, and the red cultivar, Mata Hari, could not be determined visually because salmon blotch was not visible on those cultivars. Fifty bulbs of each cultivar were sampled and attempts were made to isolate *F. proliferatum* from each. The fungus was isolated and identified morphologically from 42/50 (84%) Milky Way bulbs, 35/50 (70%) Gobi bulbs, 28/50 (56%) Mata Hari bulbs, and 21/50 Ada bulbs. The incidence of isolation for the Milky Way bulbs was higher than that of the other three cultivars, but the fungus was isolated from many bulbs that did not show any disease symptoms. Whether or not the isolates collected from the symptomatic bulbs are responsible for the disease is unknown. A 'normal' field distribution for salmon blotch of onions has not been described. The interpretation of the

field situation for this criterion led us to giving an assessment value of '1', because the disease is clearly visible only in white onion cultivars and not on yellow and red ones.

Criterion III: Weather:

The Yotvata area is hot and dry during the summer. The average temperature in July, 2012, when the onion sets were planted, was 35°C. The average annual rain fall for the Yotvata area, which is desert, is 2 mm, but the field is drip irrigated. The average temperatures in August and September were 33°C and 32°C, respectively. It is possible that the onions were under environmental stress, which could make them more susceptible to plant pathogens. In October, the average temperature was 28.8°C and in November, when the bulbs are mature, the average temperature dropped to 20°C, which is below the optimal temperature for *F. proliferatum* microconidia germination. An assessment value of '1' was assigned because weather conditions during the months of July and October were conducive for *F. proliferatum.*

Criterion IV: Temporal factors for *F. proliferatum*:

This criterion was considered in two parts; 1) Is this the usual time of year for a disease outbreak? ; 2) Is this the usual severity of symptoms for the time of year? Salmon blotch symptoms typically appear late in the growing season, when the bulbs near maturity (D. Gilette, personal communication). A value of '1' was assigned for the first part of this criterion, because every year since the disease first seen in the Yotvata field in 2008, symptoms appeared on white onion cultivars at about this time of year. The 2012 outbreak was the most severe in the white onions since the disease was first

noticed in 2008 (Gamliel, personal communication), so an assessment value of '3' was given to the second part of this criterion.

Criterion V: Field history and cultural practices:

The grower reported that he had seen salmon blotch when onions were grown in his this field previously. Before the onion sets were planted, the grower treated his field by soil solarization but did not apply fungicide like he normally would, and that could have contributed to the disease outbreak. As a result, since the outbreak under investigation could have been incited by a pathogen introduced in previous years, an assessment value of '1' was assigned.

Criterion VI: Crop rotation:

The Yotvata field farmer usually rotates onions with potatoes, maize, and sometimes fallow. Onions were planted in the Yotvata field in 2009, 2010 and 2011(D. Gilette, personal communication). It is possible that soil or plant debris remaining in the field from previous years could be the source of the 2012 outbreak fungus because *F. proliferatum* can survive on debris for several years, even though it does not produce overwintering spores. The grower did solarize his field before the onion sets were planted in 2012. An assessment value of '1' was assigned because, although the grower did not rotate onions with another crop, he did continuously plant a host that is susceptible to the fungus.

Criterion VII: Human activity:

Any farming operation will have significant human activity, and as was such the case at the Arava R&D Experiment Station, where the Yotvata field was located. During the day various vehicles, farming machinery and personnel moved in and around the production fields. Staff familiarity and the display of vehicle logos helps to assure farm security. If an unrecognized individual is seen in an unauthorized location within the experiment station, then he or she would be approached and questioned (D. Gilette, personal communication). Growers often hire extra workers, sometimes students, to help during the summers. In this case, an interview with the grower asserted that his workers were never seen doing anything suspicious and no conflicts arose between them and the grower (O. Mishli, personal communication). The Yotvata field is monitored closely during the day, but there is a possibility that individuals could gain unauthorized access at night when workers leave, despite the presence of security gates. An assessment value of '1' was given for this criterion because after interviews with the grower and experiment station manager no unusual activity was identified.

Criterion VIII: Physical evidence:

During the initial field investigation a plastic Petri plate and a commercially labeled plastic Petri dish bag were found in the cv Milky Way section of Yotvata field. Since this onion field was being used also as a research plot by scientists at the Volcani Institute, Bet Dagen, Israel, and since that research team had recently visited the field, using Petri dishes to collect samples, it was deemed highly likely that the found items had been left by them. This assumption was confirmed by questioning the researcher. An

assessment value of '1' was given because the physical evidence found in the field most likely was not related to the disease outbreak.

Criterion IX: Areas surrounding the Yotvata field:

Samples from the salt cedar windbreaks (1st perimeter) north, south, and west of the Yotvata field, along with samples of date palm seedlings (2nd perimeter) east and west of the Yotvata field, were collected. Samples from woody shrubs along the highway (3rd perimeter) also were collected. Attempts were made to isolate *F. proliferatum* from all samples. No fungus was recovered from the salt cedar samples, sixteen isolates were recovered from 117 date palm seedlings and one isolate out of 126 was recovered from vegetation collected along the highway perimeter. An assessment value of '1' was assigned because the fungus was found in vegetation adjacent to the Yotvata field.

Criterion X: Motive:

An interview with the farmer and the experiment station manager revealed no evidence of motivation to harm the grower. There was also no evidence of a politically-based attack, such as news reports of political factions or protest groups. The grower reported that all of the local growers know one another well and try to minimize competition. Onion growers in the region all purchase sets from different companies in northern Israel. They consult with each other assuring appropriate cultivar diversity at market (D. Gillette, personal communication). An assessment value of '1' was given to both subsections of this criterion.

Assessment of the Yotvata field after the lab work

A second assessment of the outbreak was performed after the results of the sample isolations and the molecular analyses were incorporated into the decision tool. This assessment was based on criteria I-XI.

Criterion IX: Surrounding areas around the Yotvata field:

DNA from the *F. proliferatum* isolates from the date palm and the highway plant samples was extracted for SSR analysis (Moncrief, 2014, chapter 4). The fungus was also isolated from volunteer weeds, including salt cedar and date palm seedlings, growing inside the 'Milky Way' portion of the Yotvata onion field. SSR profiles from the onion bulbs were clearly different from those of the date palm seedlings and phylogenetic analyses indicated that the isolates from these two substrates were separate populations (Moncrief, 2014, chapter 4). This result suggests that the date palm plantations are not the source of the fungus causing the outbreak. An assessment value of '1' was given based on the SSR results.

Criterion XI: Pathogen characteristics:

The *F. proliferatum* isolates identified morphologically in Israel were shipped to Oklahoma for further analysis. They were maintained on potato dextrose agar (PDA) plates and stored long term on sterile filter paper (Moncrief, 2014, chapter 3). Typically, *F. proliferatum* produces a dark violet pigment on PDA but we observed a range of mycelium colors including white, purple, red, green, and yellow. Usually, morphological identification is confirmed by a molecular assay such as PCR. PCR confirmation was performed with only a small number of our isolates and not done with all of them so it is

possible that some are another *Fusarium* species. DNA screening with SSR primers allowed for the characterization of isolates collected from different plant and soil populations in and around the Yotvata field (Moncrief, 2014, chapter 4). Not all of the isolate DNAs were amplified; however, the SSR results showed clear discrimination between the set isolates (from the north) and all of the isolates collected from the south (Moncrief, 2014, chapter 4). The isolates from the south, including those from the Yotvata field soil before the sets were planted, grouped with the soil and bulb isolates obtained at the time of harvest, suggesting that the pathogen was retained in the soil from previous years (Moncrief, 2014, Chapter 4). An assessment value of '1' was assigned because the SSR profiles of all of the isolates from the south were highly similar to one another and significantly different from those of the northern population.

The total point value after the early field assessment (prior to the lab results) was 33 (Table 1A). The likelihood that *F. proliferatum* was intentionally released was calculated as described by Rogers (2011). The likelihood value for this study was 35 which falls in the 'unlikely' range for assumption of a biological attack (Table 2). In the late assessment (after the incorporation of the lab results) the field assessment value was 35 (Table 1B), which is also in the 'unlikely' raenge assumption of a biological attack (Table 2).

Discussion

The decision tool analysis of the 2012 salmon blotch outbreak in onions suggests that the disease was not the result of an intentional act. A decision tool developed for the plant pathogen *WSMV* (Rogers, 2012) was modified in this study to assess its

effectiveness when applied to a different plant pathogen and cropping system. Other decision tools have been used to assess, retrospectively, if outbreaks of *Francisella tularensis* in Kosovo and the more recent *Escherichia coli* O104:H4 in Germany were due to natural causes or acts of biocrime or bioterrorism (Grunow and Finke, 2000; Radosavljevic et al. 2014).

Some of the gaps present in this study arise from the limited knowledge of the disease, salmon blotch on onions, occurring in Israel. For example, it is unknown whether or not salmon blotch has occurred in northern Israel and whether *F. proliferatum* occurs in the set field soil in the north. There are no reported descriptions of 'typical' salmon blotch disease in the field. As was observed in this study the onion cultivar may or may not have an impact on disease pattern. For example, if both white and red cultivars are planted, salmon blotch may be visible only on the white onions, even though the fungus may be present in some proportion of both cultivars. The disease pattern would be different in the two cultivars but not necessarily 'typical' in either. Furthermore, since the Yotvata field had been planted with onions for each of the three years prior to 2012, it would be useful to compare the SSR profiles of *Fp* isolates from the previous years with those we collected from the field in 2012. The field was also planted with maize prior to 2009 and it would be also interesting to know if the SSR profiles of the maize isolates are similar to those from onions, but isolates are not available from that time. If so, then the fungus could have been introduced in the maize and resided in the field during subsequent years. Probably the most important data missing is *Fp* isolates from the soil from the set fields in the north. *F. proliferatum* was recovered from the onion sets, however, and we hypothesize that isolates from the set

production fields would resemble them. If they are in fact, similar, and if the set field soil isolates do not match the production field soil isolates, then the soil from the set fields could have been the source of the fungus in the sets.

This study could be further validated by having personnel, such as other scientists, local growers and law enforcement agents in Israel use the tool during a training exercise as was done in the WSMV study (Rogers, 2011). Although the salmon blotch assessment concluded that this disease outbreak was natural, the tool should be tested also on other onion fields that are naturally infected with *F. proliferatum* (Moncrief, Chapter 4) as well as on an onion field that was intentionally inoculated with the fungus for comparison.

The effectiveness of a decision tool to investigate the issue of intentional pathogen introduction related to a disease outbreak is influenced by what information is available in published literature about the pathogen and the disease. Even the most basic biological information is helpful when determining which criteria should be chosen for a particular tool, as in a recent paper published by Radosavljevic et al. 2014, describing the development of a decision tool for assessment of the 2011 German *E. coli* O104:H4 outbreak for which the authors drew their criteria from a variety of literature sources from previous *E. coli* outbreaks in food.

The work described here confirmed the conclusion of Rogers et al. (2011) that a decision tool can be useful for assisting in a forensic investigation of a plant disease. The tool has now been tested with two plant pathogen systems, *Wheat streak mosaic virus* in wheat and *F. proliferatum* in onions, and it has the potential to be adapted for other plant pathogens and cropping systems. This tool cannot be the sole determinant of whether or

not a crime was committed, but it can help investigators focus on the criteria most appropriate for making that judgment, increasing the efficiency of their work and providing a systematic framework for determining whether the incident warrants further investigation.

Table 1A. ***Likelihood assessment based on the results prior to the lab work***

	Assessment (0-3) (A)	Weighting Factor (B)	Points (C)
I. Geographical distribution Fp is commonly found in the area	1	3	3
II. Spatial Distribution Infection pattern typical of Fp	1	2	2
III. Weather Weather conditions favorable for pathogen survival	1	3	3
IV. Temporal Usual time of year for outbreak	1	1	1
Usual severity of symptoms for time of year	3	3	9
V. Field History and cultural practices Infection found in field previously	1	1	1
VI. Crop Rotation Onion rotated with host of Fp	1	1	1
VII. Human Activity No unusual human activity present or reported	1	1	1
VIII. Physical Evidence No physical evidence found at scene	1	3	3
IX. Surrounding Areas Nearby fields, volunteer date palms, or weeds, water, fallow fields infected	1	3	3
X. Motive No motivation to harm the grower	1	3	3
No evidence of a national attack	1	3	3
XI. Pathogen Characteristics Fp strain is native to the area	0	2	0
Total			(D) **33**

Table 1: Decision tool for determining the likelihood that a *Fusarium proliferatum* outbreak could have been intentional. Each criterion has been given a weighted value. An assessment value is given to each criterion based on the situation, which is multiplied with the weighted value. The points are added up and the total point value is used to determine likelihood of intentional introduction using Table 4. *Adapted from Grunow and Finke, 2002*.

Table 1B. ***Likelihood assessment based on the results after the incorporation of the lab results***

	Assessment (0-3) (A)	Weighting Factor (B)	Points (C)
I. Geographical distribution Fp is commonly found in the area	1	3	3
II. Spatial Distribution Infection pattern typical of Fp	1	2	2
III. Weather Weather conditions favorable for pathogen survival	1	3	3
IV. Temporal Usual time of year for outbreak	1	1	1
Usual severity of symptoms for time of year	3	3	9
V. Field History and cultural practices Infection found in field previously	1	1	1
VI. Crop Rotation Onion rotated with host of Fp	1	1	1
VII. Human Activity No unusual human activity present or reported	1	1	1
VIII. Physical Evidence No physical evidence found at scene	1	3	3
X. Surrounding Areas Nearby fields, volunteer date palms, or weeds, water, fallow fields infected	1	3	3
IX. Motive No motivation to harm the grower	1	3	3
No evidence of a national attack	1	3	3
XI. Pathogen Characteristics Morphological and molecular characteristics	1	2	2
Total			(D) **35**

Table 1: Decision tool for determining the likelihood that a *Fusarium proliferatum* outbreak could have been intentional. Each criterion has been given a weighted value. An assessment value is given to each criterion based on the situation, which is multiplied with the weighted value. The points are added up and the total point value is used to determine likelihood of intentional introduction using Table 4. *Adapted from Grunow and Finke, 2002.*

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 likelihood that the criterion could be explained by natural causes.

Likelihood Worksheet

Add the points in Column B that correspond to a zero value in column A	(E) __2__
Subtract E from 36	36-(E) = (F) ___34____
Divide 36 by F	36 ÷ (F) = (G) __1.06___
Multiply total points (D) by value in G	(D) X (G) = (H) __ 35___

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	100-108
2	Likely	72-99
1	Doubtful	54-71
0	Unlikely	36-53

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

Disclaimer: Tables 1, 2, 3, 4, and the likelihood worksheet are taken from Rogers (2011). The criteria and values are changed based on the *F. proliferatu*m-onion pathosystem in Israel.

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APPENDICES

Appendix 1. *Fusarium proliferatum* isolates categorized based on phylogenetic analysis programs.

Isolate	POP	AMOVA	Bionumerics (cluster)	STRUCTURE	PCA (North or South)	UPGMA (genotype)
301	W sets	N/A	D	1	N	44
302	W sets	N/A	D	1	N	44
303	W sets	N/A	D	1	N	44
304	W sets	N/A	D	1	N	44
306	W sets	N/A	D	1	N	44
309	W sets	N/A	D	1	N	45
310	W sets	N/A	D	1	N	45
311	W sets	N/A	D	1	N	44
312	W sets	N/A	A	2	S	2
314	W sets	N/A	D	1	N	44
316	W sets	N/A	D	1	N	44
317	W sets	N/A	D	1	N	44
318	W sets	N/A	D	1	N	44
319	W sets	N/A	D	1	N	41
320	W sets	N/A	D	1	N	44
322	W sets	N/A	D	1	N	44
323	W sets	N/A	C	1	N	46
324	W sets	N/A	C	1	N	42
325	W sets	N/A	C	1	N	46
326	W sets	N/A	C	1	N	46
328	W sets	N/A	C	1	N	46
330	W sets	N/A	D	1	N	45
331	W sets	N/A	C	1	N	46
341	W sets	N/A	C	1	N	42
402	R sets	N/A	A	2	S	25
403	R sets	N/A	B	2	S	7
404	R sets	N/A	B	2	S	12
452	G sets	N/A	A	2	N	13
453	G sets	N/A	C	1	N	47
455	G sets	N/A	C	1	S	42

1002	FF weeds	N/A	A	2	S	8
1007	FF weeds	N/A	A	2	S	2
1008	FF weeds	N/A	A	2	S	9
1011	FF weeds	N/A	A	2	S	1
1014	FF weeds	N/A	A	2	S	13
1019	FF weeds	N/A	A	2	S	8
1021	FF weeds	N/A	A	2	S	8
1022	FF weeds	N/A	B	2	S	21
1024	FF weeds	N/A	B	2	S	10
1025	FF weeds	N/A	A	2	S	8
1026	FF weeds	N/A	A	2	S	8
1027	FF weeds	N/A	A	2	S	8
1028	FF weeds	N/A	A	2	S	8
1036	FF weeds	N/A	A	2	S	1
1037	FF weeds	N/A	A	2	S	13
1042	FF weeds	N/A	A	2	S	8
1048	FF weeds	N/A	A	2	S	8
1049	FF weeds	N/A	A	2	S	8
1055	FF weeds	N/A	A	2	S	13
1063	FF weeds	N/A	A	2	S	13
1064	FF weeds	N/A	A	2	S	13
1065	FF weeds	N/A	A	2	S	13
1067	FF weeds	N/A	A	2	S	13
1068	FF weeds	N/A	A	2	S	1
1071	FF weeds	N/A	B	2	S	4
1072	FF weeds	N/A	A	2	S	13
1080	FF weeds	N/A	A	2	S	13
2021	FF weeds	N/A	A	2	S	8
2022	FF weeds	N/A	A	2	S	8
2024	FF weeds	N/A	A	2	S	1
2025	FF weeds	N/A	A	2	S	8
2026	FF weeds	N/A	A	2	S	2
3065	date palms	N/A	B	2	S	29
3102	date palms	N/A	B	1:2	S	19
3106	date palms	N/A	B	2	S	37
3120	date palms	N/A	B	1	S	32
3124	date palms	N/A	B	1	S	32
3129	date palms	N/A	A	2	S	13
3135	date palms	N/A	B	1	S	34
3144	date palms	N/A	B	1	S	33
5064	HW	N/A	A	2	S	13
53	FF bulbs	N/A	A	2	S	8
54	FF bulbs	N/A	A	2	S	8
55	FF bulbs	N/A	A	2	S	8
56	FF bulbs	N/A	A	2	S	8
58	FF bulbs	N/A	A	2	S	8
59	FF bulbs	N/A	A	2	S	2
60	FF bulbs	N/A	A	2	S	13
61	FF bulbs	N/A	A	2	S	30

62	FF bulbs	N/A	A	2	S	27
63	FF bulbs	N/A	B	2	S	31
64	FF bulbs	N/A	B	2	S	10
65	FF bulbs	N/A	A	2	S	2
75	FF bulbs	N/A	A	2	S	8
77	FF bulbs	N/A	B	2	S	5
79	FF bulbs	N/A	A	2	S	8
80	FF bulbs	N/A	A	2	S	1
84	FF bulbs	N/A	C	2	S	40
87	FF bulbs	N/A	A	2	S	2
94	FF bulbs	N/A	A	2	S	13
95	FF bulbs	N/A	A	2	S	26
100	FF bulbs	N/A	A	2	S	1
117	FF bulbs	N/A	B	2	S	21
118	FF bulbs	N/A	A	2	S	16
119	FF bulbs	N/A	B	2	S	18
120	FF bulbs	N/A	B	2	S	22
161	FF bulbs	N/A	A	2	S	17
176	FF bulbs	N/A	C	1	S	40
181	FF bulbs	N/A	A	2	S	13
701	C bulbs	N/A	A	2	S	13
703	C bulbs	N/A	A	2	S	13
712	C bulbs	N/A	A	2	S	13
713	C bulbs	N/A	C	1	S	47
716	C bulbs	N/A	A	2	S	13
717	C bulbs	N/A	A	2	S	13
721	C bulbs	N/A	A	2	S	13
726	C bulbs	N/A	A	2	S	13
728	C bulbs	N/A	A	2	S	13
731	C bulbs	N/A	A	2	S	13
732	C bulbs	N/A	B	1:2	S	20
736	C bulbs	N/A	A	2	S	13
737	C bulbs	N/A	A	2	S	1
746	C bulbs	N/A	A	2	S	13
751	C bulbs	N/A	A	2	S	13
758	C bulbs	N/A	A	2	S	13
763	C bulbs	N/A	A	2	S	13
767	C bulbs	N/A	A	2	S	13
776	C bulbs	N/A	A	2	S	13
778	C bulbs	N/A	A	2	S	13

902	FoD bulbs	N/A	A	2	S	13
904	FoD bulbs	N/A	B	2	S	22
911	FoD bulbs	N/A	A	2	S	13
914	FoD bulbs	N/A	A	2	S	13
916	FoD bulbs	N/A	A	1	S	43
922	FoD bulbs	N/A	A	2	S	13
926	FoD bulbs	N/A	C	1	S	42
942	FoD bulbs	N/A	B	2	S	24
943	FoD bulbs	N/A	A	2	S	1
949	FoD bulbs	N/A	A	2	S	1
505	g bulbs	N/A	A	2	S	8
507	g bulbs	N/A	A	2	S	13
510	g bulbs	N/A	A	2	S	13
513	g bulbs	N/A	B	2	S	18
514	g bulbs	N/A	A	2	S	8
519	g bulbs	N/A	A	2	S	13
526	g bulbs	N/A	A	2	S	13
S23	FF soil	N/A	D	1	S	35
S48	FF soil	N/A	B	1	S	38
S65	FF soil	N/A	A	2	S	6
S66	FF soil	N/A	A	2	S	6
S68	FF soil	N/A	A	2	S	1
S70	FF soil	N/A	A	2	S	13
S71	FF soil	N/A	A	2	S	13
S76	FF soil	N/A	A	2	S	13
S81	FF soil	N/A	D	1	S	43
S102	FF soil	N/A	B	2	S	22
S103	FF soil	N/A	A	2	S	3
S110	FF soil	N/A	A	2	S	13
S121	FF soil	N/A	A	2	S	13
S125	FF soil	N/A	A	2	S	13
S127	FF soil	N/A	A	2	S	13
S133	FF soil	N/A	A	2	S	13
S135	FF soil	N/A	C	1	S	42
S138	FF soil	N/A	B	2	S	18
S143	FF soil	N/A	C	1	S	49
S147	FF soil	N/A	A	2	S	14
S149	FF soil	N/A	A	2	S	14
S157	FF soil	N/A	A	2	S	13
S158	FF soil	N/A	B	2	S	23
S162	FF soil	N/A	A	2	S	13
S183	FF soil	N/A	A	2	S	13
S192	FF soil	N/A	D	2	S	43
S199	FF soil	N/A	A	2	S	13

S713	C soil	N/A	A	2	S	13
S718	C soil	N/A	B	2	S	22
S721	C soil	N/A	A	2	S	13
S726	C soil	N/A	A	2	S	13
S741	C soil	N/A	A	2	S	1
S748	C soil	N/A	A	2	S	13
S761	C soil	N/A	A	2	S	1
S762	C soil	N/A	A	2	S	13
S768	C soil	N/A	A	2	S	13
S772	C soil	N/A	A	2	S	13
S776	C soil	N/A	A	2	S	1
S737	C soil	N/A	A	2	S	13
S753	C soil	N/A	A	2	S	13
S757	C soil	N/A	A	2	S	13
S777	C soil	N/A	A	2	S	13
S704	C soil	N/A	B	2	S	39
S722	C soil	N/A	A	2	S	1
S728	C soil	N/A	B	2	S	15
S727	C soil	N/A	B	2	S	10
S746	C soil	N/A	A	2	S	13
S751	C soil	N/A	A	2	S	13
S766	C soil	N/A	B	2	S	48
S743	C soil	N/A	A	2	S	13
S503	g soil	N/A	A	2	S	27
S506	g soil	N/A	A	2	S	28
S509	g soil	N/A	A	2	S	13
S511	g soil	N/A	A	2	S	13
S512	g soil	N/A	B	2	S	22
S513	g soil	N/A	A	2	S	25
S522	g soil	N/A	A	2	S	8
S527	g soil	N/A	A	2	S	13
S529	g soil	N/A	A	2	S	8
S532	g soil	N/A	A	2	S	8
S536	g soil	N/A	A	2	S	1
S538	g soil	N/A	A	2	S	13
S541	g soil	N/A	A	2	S	11
S543	g soil	N/A	A	2	S	28
S547	g soil	N/A	A	2	S	13
S549	g soil	N/A	A	2	S	8
S531	g soil	N/A	A	2	S	13

S537	g soil	N/A	A	2	S	13
S859	FF soil BP	N/A	A	2	S	8
S885	FF soil BP	N/A	A	2	S	13
S889	FF soil BP	N/A	A	2	S	1
S852	FF soil BP	N/A	A	2	S	13
S856	FF soil BP	N/A	A	2	S	13
S912	FoD soil	N/A	A	2	S	8
S917	FoD soil	N/A	A	2	S	28
S938	FoD soil	N/A	B	1	S	36
S921	FoD soil	N/A	A	2	S	13
S922	FoD soil	N/A	A	2	S	28
S923	FoD soil	N/A	A	2	S	13
S924	FoD soil	N/A	A	2	S	8

Appendix 2. All *Fusarium proliferatum* isolates from Israel, Germany, Austria, and North America with their DNA concentrations and amplicon sizes for each SSR primer. Isolates in yellow: DNA concentration is unknown. Isolates in dark orange: No amplification with one or more SSR primers.

Name	Nanodrop (ng/ul)	sample	SSR38	SSR45	SSR92	SSR68	SSR109	SSR18
Fp48	9.5	FF bulb						
Fp181	8	FF bulb	381	140	349	111	393	372
Fp74	8.6	FF bulb						
Fp164	11.2	FF bulb						
Fp69	8.8	FF bulb						
Fp146	11.7	FF bulb						
Fp124	8	FF bulb						
Fp71	8.4	FF bulb						
Fp144	8	FF bulb						
Fp90	8.9	FF bulb						
Fp43	10	FF bulb						
Fp176	10.2	FF bulb	384	140	360	116	397	372
Fp89	8.9	FF bulb						
Fp72	9.7	FF bulb						
Fp165	18.5	FF bulb						
Fp88	9	FF bulb						
Fp131	8	FF bulb						
Fp128	6.4	FF bulb						
Fp133	7.1	FF bulb						
Fp75	8.7	FF bulb	381	140	349	111	393	373
Fp69	7.6	FF bulb						
Fp73	7.5	FF bulb						
Fp68	10.3	FF bulb						
Fp71	10.8	FF bulb						
Fp48	10.5	FF bulb						
Fp88	13.5	FF bulb						
Fp55	8.8	FF bulb	381	140	349	111	393	373
Fp92	10.2	FF bulb						
Fp67	7.8	FF bulb						
Fp91	9.6	FF bulb						
Fp89	11	FF bulb						
Fp74	8.3	FF bulb						
Fp19	6.2	FF bulb						
Fp46	7.5	FF bulb						

Fp72	7.8	FF bulb						
Fp62	7.2	FF bulb	381	141	349	112	393	372
Fp57	8.9	FF bulb					393	
Fp43	6.1	FF bulb						
FP53	13.5	FF bulb	381	140	349	111	393	373
FP61	12.4	FF bulb	381	141	348	112	393	373
FP100	5.5	FF bulb	381	140	348	111	393	372
FP64	8.3	FF bulb	381	140	349	116	393	373
FP56	6.5	FF bulb	381	140	349	111	393	373
FP54	8.5	FF bulb	381	140	349	111	393	373
FP85	7.7	FF bulb						
FP96	8.6	FF bulb						
FP58	12.5	FF bulb	381	140	349	111	393	373
FP51	14.6	FF bulb						
FP65	23.6	FF bulb	381	140	348	111	393	373
FP79	6.7	FF bulb	381	140	349	111	393	373
FP77	9.5	FF bulb	386	140	348	111	393	372
FP95	3.4	FF bulb	381	141	348	112	393	372
FP66	9.8	FF bulb						
FP84	7.9	FF bulb	384	140	360	116	397	372
FP70	36.7	FF bulb						
FP63	41.3	FF bulb	381	141	348	117	393	373
FP73	7.2	FF bulb						
FP80	11.3	FF bulb	381	140	348	111	393	372
FP60	10.3	FF bulb	381	140	349	111	393	372
FP87	9.8	FF bulb	381	140	348	111	393	373
FP81	7.1	FF bulb						
FP94	5	FF bulb	381	140	349	111	393	372
FP52	16.7	FF bulb						
Fp161	206.5	FF bulb	381	140	349	111	393	364
FP164	64.5	FF bulb	381	140	349	111	393	
FP107	47.3	FF bulb	384	140	349	111	393	
FP118	55.9	FF bulb	381	140	349	111	393	374
FP119	99.8	FF bulb	381	140	349	116	393	372
FP120	193.8	FF bulb	384	140	349	111	393	372
FP176	61.8	FF bulb	384	140	360	116	397	372
FP181	102.1	FF bulb	381	140	349	111	393	372
FP122	44.5	FF bulb					393	
FP196	12.4	FF bulb						
FP111	19.9	FF bulb						
FP117	17.8	FF bulb	384	141	349	111	393	372
FP301	10.9	W sets	384	145	360	116	397	373
FP302	11.4	W sets	384	145	360	116	397	373
FP303	21.5	W sets	384	145	360	116	397	373
FP304	13.9	W sets	384	145	360	116	397	373
FP305	10.7	W sets						
FP306	9	W sets	384	145	360	116	397	373
FP307	10.5	W sets						
FP309	7.7	W sets	384	144	360	116	397	373
FP310	18.8	W sets	384	144	360	116	397	373
FP311	14.6	W sets	384	145	360	116	397	373

FP312	11.7	W sets	381	140	348	111	393	373
FP313	12.7	W sets						
FP314	15.3	W sets	384	145	360	116	397	373
FP315	14	W sets						
FP316	19.7	W sets	384	145	360	116	397	373
FP317	16.4	W sets	384	145	360	116	397	373
FP318	11	W sets	384	145	360	116	397	373
FP319	14.9	W sets	384	145	360	116	397	372
FP320	23.9	W sets	384	145	360	116	397	373
FP321	18.9	W sets						
FP322	12.4	W sets	384	145	360	116	397	373
FP324	8	W sets	384	141	360	116	397	372
FP325	8	W sets	384	141	360	116	397	373
FP326	8.8	W sets	384	141	360	116	397	373
FP327	7.1	W sets						372
FP328	12.3	W sets	384	141	360	116	397	373
FP329	11	W sets						
FP330	20.5	W sets	384	144	360	116	397	373
FP331	9.2	W sets	384	141	360	116	397	373
FP341		W sets	384	141	360	116	397	372
FP342		W sets	384		360			
FP452	9.1	G sets	381	140	349	111	393	372
FP453	8	G sets	384	140	360	116	397	373
FP454	6.4	G sets					393	373
FP455	7.9	G sets	384	141	360	116	397	372
FP401	8.1	R sets						
FP402	7.6	R sets	381	141	348	111	393	372
FP403	12.9	R sets	387	140	348	111	393	372
FP404	22	R sets	387	140	349	111	393	373
FP1014	28.5	FF weeds	381	140	349	111	393	372
FP1018	109.5	FF weeds					393	
FP1019	30.9	FF weeds	381	140	349	111	393	373
FP1021		FF weeds	381	140	349	111	393	373
FP1022		FF weeds	384	141	349	111	393	372
FP1026	21.6	FF weeds	381	140	349	111	393	373
FP1029	87.5	FF weeds						
FP1034	53.9	FF weeds						372
FP1036	58.5	FF weeds	381	140	348	111	393	372
FP1045	11.1	FF weeds			349			
FP1047	56.6	FF weeds					393	
FP1049	23.8	FF weeds	381	140	349	111	393	373
FP1035	74	FF weeds			349			372
FP1043	32.6	FF weeds						
FP1002	98	FF weeds	381	140	349	111	393	373
FP1008	65.9	FF weeds	381	140	349	111	394	373
FP1033	136.4	FF weeds						
FP1011	34.9	FF weeds	381	140	348	111	393	372
FP1012	54.2	FF weeds						
FP1042	49	FF weeds	381	140	349	111	393	373
FP1028	48.6	FF weeds	381	140	349	111	393	373
FP1007	51.6	FF weeds	381	140	348	111	393	373

FP1025	147.4	FF weeds	381	140	349	111	393	373
FP1024	64.4	FF weeds	381	140	349	116	393	373
FP1048	35.8	FF weeds	381	140	349	111	393	373
FP1037	34	FF weeds	381	140	349	111	393	372
FP1055		FF weeds	381	140	349	111	393	372
FP1064		FF weeds	381	140	349	111	393	372
Fp1065	80.6	FF weeds	381	140	349	111	393	372
Fp1072	67.7	FF weeds	381	140	349	111	393	372
FP1066	80	FF weeds						
FP5907	33.6	HW						
FP1055	123.9	FF weeds	381	140	349	111	393	372
FP1027	95.8	FF weeds	381	140	349	111	393	373
FP5064	15.3	HW	381	140	349	111	393	372
FP1901	82.1							
FP1063	29.3	FFweeds	381	140	349	111	393	372
FP1070	26.5	FFweeds						
FP1071	37.5	FFweeds	384	140	348	111	393	372
FP1067	14.9	FFweeds	381	140	349	111	393	372
FP1068		FFweeds	381	140	348	111	393	372
FP2026	8.4	FFweeds	381	140	348	111	393	373
FP2025	10.2	FFweeds	381	140	349	111	393	373
FP2021	7.8	FFweeds	381	140	349	111	393	373
FP2024	10.6	FFweeds	381	140	348	111	393	372
FP2022	7.1	FFweeds	381	140	349	111	393	373
FP3102	18.1	date palms	396	140	349	116	393	372
FP3012	16.7	date palms						
FP3029	22.6	date palms						
FP3041	13.6	date palms						
FP3052	11.8	date palms						
FP3065	13.8	date palms	378	140	349	94	409	372
FP3102	11.2	date palms	396	140	349	116	393	372
FP3106	15.5	date palms	396	140	349	112	396	395
FP3113	11.1	date palms	399	140	349			
FP3120	13.7	date palms	390	140	349	116	394	373
FP3123	12.9	date palms	387	140	349			
FP3124	13.9	date palms	390	140	349	116	394	373
FP3129	17	date palms	381	140	349	111	393	372
FP3135	9.4	date palms	402	140	349	116	390	373
FP3136	10.4	date palms						
FP3139	15.8	date palms						
FP3144	15.2	date palms	390	140	349	116	394	372
Fp505	90.8	G bulb	381	140	349	111	393	373
Fp507		G bulb	381	140	349	111	393	372
Fp508	125.7	G bulb	381	140	348			
Fp509	97.4	G bulb	381	140	349			
Fp510	44.5	G bulb	381	140	349	111	393	372
Fp512	40.6	G bulb	381	140	349		393	
Fp513	465.1	G bulb	381	140	349	116	393	372
Fp514	20.5	G bulb	381	140	349	111	393	373
Fp519	210.3	G bulb	381	140	349	111	393	372
Fp535	23	G bulb	381	140	348			

Fp536	35.2	G bulb				111		
Fp526		G bulb	381	140	349	111	393	372
Fp537	76.4	G bulb	381	140	349	111	393	
FP539	43.5	G bulb	381	140	349			
FP540		G bulb	381	140	349		393	
Fp701		G bulb	381	140	349	111	393	372
Fp703		G bulb	381	140	349	111	393	372
Fp 713		C bulb	384	140	360	116	397	373
Fp702	29	C bulb				111	393	372
Fp726	83.3	C bulb	381	140	349	111	393	372
S727		C bulb				111	393	
Fp728		C bulb	381	140	349	111	393	372
Fp731		C bulb	381	140	349	111	393	372
Fp732		C bulb	384	140	349	116	393	372
Fp778	98.8	C bulb	381	140	349	111	393	372
FP767	60.7	C bulb	381	140	349	111	393	372
FP766	47.2	C bulb	381	140	349			
FP703	106.4	C bulb	381	140	349	111	393	372
FP763	19	C bulb	381	140	349	111	393	372
Fp751		C bulb	381	140	349	111	393	372
FP721	53.7	C bulb	381	140	349	111	393	372
FP758	33.7	C bulb	381	140	349	111	393	372
FP712	16.4	C bulb	381	140	349	111	393	372
FP711	43.4	C bulb	381	140	349	111	393	
FP746	23.1	C bulb	381	140	349	111	393	372
FP716	59.6	C bulb	381	140	349	111	393	372
FP776	35.9	C bulb	381	140	349	111	393	372
FP717	29	C bulb	381	140	349	111	393	372
FP733	14.1	C bulb						
FP723	64.5	C bulb				111	393	372
FP707	64.7	C bulb	381	140	348			
FP747	99.3	C bulb						
FP736		C bulb	381	140	349	111	393	372
FP708	19.8	C bulb						
FP737	12	C bulb	381	140	348	111	393	372
Fp926	85.1	FoD bulb	384	141	360	116	397	372
FP924	54.8	FoD bulb	381	140	348			
FP941	45.9	FoD bulb						
FP928	97.7	FoD bulb	393	140	349			
FP922	25.9	FoD bulb	381	140	349	111	393	372
FP948	95.8	FoD bulb						
FP942	37.9	FoD bulb	384	140	349	111	400	372
FP908	9.1	FoD bulb	381	140	349			
FP911	49.1	FoD bulb	381	140	349	111	393	372
FP902	17.8	FoD bulb	381	140	349	111	393	372
Fp914	40.3	FoD bulb	381	140	349	111	393	372
FP904	17.3	FoD bulb	384	140	349	111	393	372
S23		FF soil	378	144	352	116	398	373
S48		FF soil	369	145	352	117	393	372
S156	40	FF soil				111	393	
S153	80.1	FF soil						373

S159	56.5	FF soil				148	393	
S147	73	FF soil	380	140	349	111	393	372
S158	54.8	FF soil	384	140	349	111	393	371
S110	36.7	FF soil	381	140	349	111	393	372
S107	78.5	FF soil				116	397	
S121	107.1	FF soil	381	140	349	111	393	372
S125	141.7	FF soil	381	140	349	111	393	372
S122	3.9	FF soil				111	393	372
S127	6.5	FF soil	381	140	349	111	393	372
S711	50.8	FF soil				116	393	
S171	92	FF soil						
S160	52	FF soil				111	393	
S70	78.7	FF soil	381	140	349	111	393	372
S95	50.2	FF soil				111	393	372
S103	27.9	FF soil				116	393	
S143		FF soil	383	143	360	115	397	372
S144	19.6	FF soil				111	393	
S71	60.9	FF soil						
S123	74.1	FF soil					393	372
S150	7.1	FF soil						372
S141	53.9	FF soil				111	393	
S147		FF soil	380	140	349	111	393	372
S138	5.2	FF soil	381	140	349	116	393	372
S157	11	FF soil	381	140	349	111	393	372
S142	11.3	FF soil				111	393	
S149	4.3	FF soil	380	140	349	111	393	372
S65		FF soil	380	140	348	111	393	372
S66	83.8	FF soil	380	140	348	111	393	372
S65	20.7	FF soil	380	140	348	111	393	372
S135	66.8	FF soil	384	141	360	116	397	372
S90	98.3	FF soil						372
S92		FF soil				111	393	
S93	115.6	FF soil				111	393	
S67	16.1	FF soil						
S97	117.4	FF soil						
S71		FF soil	381	140	349	111	393	372
S72	18.7	FF soil				111	393	
S68	34.9	FF soil	381	140	348	111	393	372
S99	5.5	FF soil				111	393	
S76	182.9	FF soil	381	140	349	111	393	372
S133	161.5	FF soil	381	140	349	111	393	372
S162	56.3	FF soil	381	140	349	111	393	372
S81	84.7	FF soil	384	144	360	116	397	372
S192	95.5	FF soil	384	144	360	116	397	372
S178	31.7	FF soil	381	140	349			
S183	10.5	FF soil	381	140	349	111	393	372
S115	120.3	FF soil						
S102	129	FF soil	384	140	349	111	393	372
S103		FF soil	381	140	348	116	393	372
S199	59	FF soil	381	140	349	111	393	372
S503		G soil	381	141	349	112	393	372

S504		G soil	381	140	349			
S506		G soil	381	141	349	111	393	372
S509		G soil	381	140	349	111	393	372
S512	82	G soil	384	140	349	111	393	372
S513		G soil	381	141	348	111	393	372
S519		G soil	381	140	349			
S534	22.3	G soil						
S578	57.8	G soil						
S545	91.9	G soil	381	140	349			
S547	54.8	G soil	381	140	349	111	393	372
S529		G soil	381	140	349	111	393	373
S530	21.5	G soil						
S531	24.7	G soil	381	140	349	111	393	372
S537	32.6	G soil						
S538		G soil	381	140	349	111	393	372
S522	47.8	G soil	381	140	349	111	393	373
S527	74.7	G soil	381	140	349	111	393	372
S532	36.6	G soil	381	140	349	111	393	373
S536	39.3	G soil	381	140	348	111	393	372
S541		G soil	381	141	349	111	393	373
S542	82.5	G soil						
S543		G soil	381	141	349	111	393	372
S545	76.6	G soil						
S549	26.1	G soil	381	140	349	111	393	373
S741	13.4	C soil	381	140	348	111	393	372
S726	54.8	C soil	381	140	349	111	393	372
S748	36.4	C soil	381	140	349	111	393	372
S738	60	C soil				111	393	372
S711	50.8	C soil				111	393	
S773	98	C soil				111	393	
S776	65.3	C soil	381	140	348	111	393	372
S713	39.4	C soil	381	140	349	111	393	372
S718	103.6	C soil	384	140	349	111	393	372
S712	23.3	C soil				111	393	372
S753		C soil	381	140	349	111	393	372
S758	48.9	C soil				111		
S763	167.9	C soil						
S761	53.7	C soil	381	140	348	111	393	372
S771	155.8	C soil						
S706	80.9	C soil	381	140	349			
S721	12.3	C soil	381	140	349	111	393	372
S762	58.6	C soil	381	140	349	111	393	372
S772	23	C soil	381	140	349	111	393	372
S768	99.6	C soil	381	140	349	111	393	372
S704	62.3	C soil	372	144	351	116	393	372
S708	11.5	C soil						
S737	52.7	C soil	381	140	349	111	393	372
S722		C soil	381	140	348	111	393	372
S723	39.8	C soil	394	140	349			
S757	10.1	C soil	381	140	349	111	393	372
S777	37.8	C soil	381	140	349	111	393	372

S703	75.9	C soil						
S727	16.7	C soil	381	140	349	116	393	373
S728	25.4	C soil	393	140	349	111	393	372
S751	55	C soil	381	140	349	111	393	372
S766	11.3	C soil	384	140	360	116	393	373
S746	27.4	C soil	381	140	349	111	393	372
S742	14	C soil	381	140	348			
S743	62.8	C soil	381	140	349	111	393	372
S702	12.8	C soil						
S701	7.5	C soil	384	140	360			
FpS728	46.6	C soil						
FpS732	70.5	C soil						
S912	34.8	FoD soil	381	140	349	111	393	373
S917		FoD soil	381	141	349	111	393	372
S928	58.6	FoD soil						
S938	85.2	FoD soil	378	140	352	116	393	373
S921		FoD soil	381	140	349	111	393	372
S922		FoD soil	381	141	349	111	393	372
S923	75.8	FoD soil	381	140	349	111	393	372
S924	66.7	FoD soil	381	140	349	111	393	373
S926	134.8	FoD soil						
S936	173.6	FoD soil						
S947		FoD soil	381	140	348			
S859	31.9	FF soil before	381	140	349	111	393	373
S882	63.5	FF soil before	381	140	349			
S885	95.4	FF soil before	381	140	349	111	393	372
S852	78.9	FF soil before	381	140	349	111	393	372
S856	72.6	FF soil before	381	140	349	111	393	372
S889	107.9	FF soil before	381	140	348	111	393	372
S862	47.6	FF soil before						
Fp86M	18.2	Germany	384	145	352	116	393	
Fp24C	30.7	Germany	369	145	352	116	393	
Fp21C	20.6	Germany	378	145	351	116	398	
Fp13A	27.4	Germany				117	393	
Fp60E	24.7	Germany	375	145	349	117	401	
Fp200S	22.2	Germany						
Fp35C	14.9	Germany	384	145	352	116	393	
Fp17L	17.2	Germany	384	145	352	116	393	
Fp69S	22.4	Germany						
Fp34B	12.9	Germany				112	393	
Fp2B	4.7	Germany	369	145	351	117	393	
Fp3B	1.1	Germany	381	141	348			
Fp11F	41.4	Germany						
Fp26I	20	Germany	375	145	349	117	393	
Fp227S	22.2	Germany						
Fp67M	7.2	Germany	381	141	349	112	393	
Fp1-c-o	27.9	Germany	405	141	349	116	390	
Fp44G	33.6	Germany	384	141	349	112	393	
Fp20J	25.3	Germany						
Fp41A	49.5	Germany	381	141	349	112	393	
FpZ2	15.4	Germany	381	141	349	112	393	

Fp90M	33.4	Germany	381	141	349	112	393	
FpO-9-1	29.6	Germany						
Fp219S	34.5	Germany						
Fp222D	21.5	Germany						
Fp82M	29.9	Germany	381	141	348	112	393	
Fp7A	26.1	Germany	405	141	349	116	394	
Fp14F	54.5	Germany	381	141	348	112	393	
Fp91M	78.6	Germany	381	141	349	112	393	
Fp13B	3	Germany	368	141	334	112	394	
FpCO4	7.8	Germany	384	141	349			
Fp32E	45.3	Germany	384	145	352	116	393	
Fp15H	15	Germany	381	141	348	112	393	
Fp37E	12.7	Germany				112	394	
Fp420	14.8	Germany						
Fp15Z	8.6	Germany	384	145	360	116	397	
Fp31E	2.3	Germany				112	394	
Fp21Z	15.9	Germany	384	145	360	116	397	
Fp5Z	20.1	Germany						
FP259S	15.2	Germany	372	145	352	117	403	
FP78M	21.1	Germany				112	393	
FP395S	36	Germany				116	396	
FP163mais	44.3	Germany	384	141	349	112	393	
FP46D	20.7	Germany	387	141	349	112	393	
FP2K	30.5	Germany	384	145	349	116	393	
FP25A	27.2	Germany	381	141	349	112	393	
FP510S	23.2	Germany	406	141	349	117	390	
FP19H	33.8	Germany						
FP94M	48.7	Germany	381	141	349	111	393	
FP110L	56.5	Germany						
FP43B	51.6	Germany	384	141	348	112	393	
FP49C	28.1	Germany	384	141	349	111	393	
FP30H	22.7	Germany						
FP29mais	23.6	Germany	381	141	348	111	393	
FP29E	38.7	Germany				116	396	
FP241S	36	Germany	372	145	351			
Fp56E	11.8	Germany						
KSU_2549	21.7	USA						
KSU_2347	33	USA				117	401	372
KSU_667	37	USA						
KSU_2825	27.8	USA						
KSU_2238	16.2	USA	384	141	349	112	393	372
KSU_2371	22.4	USA	384	141	348			
KSU_1119	17.7	USA	372	141	349	117	396	373
KSU_517	16.5	USA						
KSU_666	24.9	USA	384		349			
KSU_436	42.6	USA	381	141	348			
FP1272	56.3	USA	375	145	349	117	401	372
FP1275	21.2	USA						
FP598	33.9	USA	375	145	349	117	401	372
FP526	23.2	USA						
FP1126	31.9	USA	375	145	349			

FP2294	29.6	USA	381	141	349	112	393	373
FP797	35.2	USA	393	141	348			
FP791	35.3	USA	378	141	349			
FP457	40	USA						
FP665	44.3	USA	399	145	349	117	406	372
FP661	35.5	USA						
FP1280	149.8	USA	375	145	351			
FP591	30.7	USA				117	396	372
Fp678	21.9	USA						
FP431		USA	381	141	348			
FP593		USA	381	141	349			
FP598		USA						
FP1944		USA	363	171	352			
FP1507		USA						
FP1259		USA	375	145	351			
FP2208		USA	384	141	348	112	393	372
FP2356		USA	381	141	349	112	393	372
FP663		USA	369	141	349			
FP650		USA						
FP2373		USA	381	141	352	112	392	372
FP506		USA	381	141	348	112	393	372
FP640		USA	363	166	352			
FP2339		USA						
FP1275		USA						
FP652		USA	369	141	348			
FP791		USA	411	141	348	117	393	372
FP526		USA	402	141	351	117	402	372
FP661		USA						
FP797		USA						
FP1280		USA						
FP566		USA	378	141	352	116	393	372
FP638		USA	372	141	349	117	396	372
FP682		USA	375	145	349	117	401	373
FP1932		USA	363	171	352	117	391	372
FP1929		USA	363	140	352			
FP1276		USA	387	140	349	112	393	372
FP1174		USA	372	141	349	117	393	372
FP2234		USA	381	141	349	117	393	372
FP499		USA	384	141	349	112	393	372
FP830		USA	384	141	349	117	394	372
FP2244		USA	384	141	349			
FP1508		USA	378	141	349	117	406	372
FP2227		USA	384	145	360			
FP656		USA	378	141	349			
FP662		USA	369	145	349			

VITA

Ian Russell Moncrief

Candidate for the Degree of

Doctor of Philosophy

Thesis: VALIDATING FORENSIC TOOLS FOR CROP BIOSECURITY: CASE STUDY INVESTIGATION OF SALMON BLOTCH OF ONIONS IN ISRAEL

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